University of Alberta

The Effects of Hypoxia, and Reoxygenation With 21% and 100% Oxygen in the Newborn Liver

by

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To Sandy
For all her love, patience and support

ABSTRACT

Neonatal asphyxia and the subsequent reoxygenation can cause severe tissue injury. We hypothesized that reoxygenation with low oxygen concentrations would be as efficacious as with100% oxygen in restoring hepatic perfusion and oxygen metabolism, with no increase in tissue injury. A piglet model of neonatal asphyxia was developed for the assessment of hepatic hemodynamics, oxygen metabolism, oxidative stress and tissue injury. The 1 – 3 day old piglets underwent 2 hours of severe hypoxia followed by reoxygenation with 21% or 100% oxygen. The piglets reoxygenated with 21% oxygen showed equivalent recovery of hemodynamic parameters and oxygen metabolism, but without the increased oxidative stress of those given 100% oxygen. There were increased hepatic tissue lactate levels in the 21% group but no evidence of increased hepatocellular injury.

It is concluded that that the use of 21% oxygen is safe and effective in restoring physiological parameters of hepatic function after neonatal asphyxia and avoids excessive oxidative stress.

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ABBREVIATIONS

ADP – Adenosine 5' diphposphate

AMP – Adenosine 5' monophosphate

ANOVA – Analysis of variance

ALT – Alanine transaminase

AST – Aspartate transaminase

ATP – Adenosine 5' triphosphate

BP – Blood pressure

CI – Cardiac index

CVP – Central venous pressure

DNA – Deoxyribonucleic acid

DO_2 - Oxygen delivery

ECG – Electrocardiogram

EO_2 – Oxygen extraction

FiO_2 – Fractional inspired oxygen concentration

Fr – French sizing of catheters

GSH – Reduced glutathione

GSSG – Oxidized glutathione

GTP – Guanine triphosphate

HA – Hepatic artery

HA FI – Hepatic arterial flow index

HPF – High power field

H-R - Hypoxia and reoxygenation

IAP – Inhibitor of apoptosis proteins

I-R – Ischemia-Reperfusion

IV – Intravenous

LDH – Lactate dehydrogenase

LSD – Least squares difference

MDA – Malondialdehyde

MMP - Matrix metalloproteinase

MPT – Mitochondrial membrane permeability transition

NAC – N-Acetyl-cysteine

NAD^+ - Oxidized nicotinamide adenine dinucleotide

NADH – Reduced nicotinamide adenine dinucleotide

NADPH – Reduced nicotinamide adenine dinucleotide phosphate

$NADP^+$ - Oxidized nicotinamide adenine dinucleotide phosphate

NF-κB - A transcription factor

NO – Nitric oxide

PA – Pulmonary artery

p_aCO_2 – Arterial partial pressure of carbondioxide

p_aO_2 - Arterial partial pressure of oxygen

PAF – Platelet activating factor

PV – Portal vein

PVFI – Portal venous flow index

ROH, $ROO^{\cdot}$ R – Lipid, protein or polysaccharide residue

ROS – Reactive oxygen species

RNS – Reactive nitrogen species

S_aO_2 – Arterial hemoglobin oxygen saturation

SEC – Sinusoid endothelial cell

SOD – Superoxide dismutase

THFI – Total hepatic flow index

TNF-α - Tumor necrosis factor α

UVC – Umbilical vein catheter

VO_2 – Oxygen consumption

XDH – Xanthine dehydrogenase

XO – Xanthine oxidase

Chapter 1

Hypoxia-reoxygenation induced organ injury in the newborn: How do hypoxia, oxygen and free radicals affect the liver?

Hepatic H-R and free radicals

1. Clinical aspects of perinatal asphyxia, free radicals and oxygen

The newborn infant has complex mechanisms in place to manage the transition from a relatively oxygen-poor environment in utero to an oxygen-rich one after birth. While some defences of newborn infants against such oxidative stress are less than later in life,[1] others seem well developed.[2] The cellular mechanisms to limit oxidative stress include gene regulation at the transcription level controlled by the redox state of cells;[3] mitochondrial, cytosolic and membrane directed antioxidant mechanisms;[4-8] serum antioxidants[1] and signalling mediated by reactive oxygen species (ROS).[7,9] Hypoxia and ischemia during perinatal asphyxia, disrupts this transition. Profound changes in cellular function occur during hypoxia, which can be protective to the cell and organ.[10,11] If a point of decompensation is reached, then cellular recovery from hypoxia may not occur or, in a cell that is still viable, antioxidant mechanisms may not be adequate to buffer the additional stress of reoxygenation.

The assessment and treatment of injury resulting from perinatal asphyxia is rudimentary. The clinical assessment of injury of the hypoxic neonate is good at identifying and predicting the outcome of very severe, and very mild cerebral insults.[12] The moderate insults seem to have very variable clinical outcomes however. Specific therapies, other than general supportive treatment, are few.[13] Hypothermia is one developing strategy, which may ameliorate moderate insults.[14] Despite the obvious implications of acute cerebral injury for long term outcome, the insult from perinatal asphyxia is multi-organ, and other organ responses have been less well studied than the brain.

The liver seems less susceptible to clinically detectable injury than other organs.[15,16] However, liver transaminases are raised in infants following perinatal asphyxia,[17,18] and these correlate with the development of hypoxic ischemic encephalopathy.[19] Persisting cholestasis may occur if there is an associated hepatitis[20] and in severe cases of asphyxia, coagulation is deranged.[21-23] At post-mortem, the liver of asphyxiated infants shows histopathologic changes, although these changes may not correlate with the clinical grading of asphyxia.[21] Effects on organs downstream from the liver have also been described in animals during reoxygenation.[24]

Studies of neonatal asphyxial injury have confirmed the involvement of reactive species, particularly the ROS, reactive nitrogen species and iron.[25-27] High levels of non-protein bound iron predispose to excess free radical production.[28] Reactive species effects are apparent in birth asphyxia by 24 hours of age. Asphyxiated infants have increased lipid peroxidation, as measured by malondialdehyde levels, and increased activity of the antioxidant enzymes superoxide anion dismutase (SOD) and catalase, compared to normal term infants.[29] Increased non-protein bound iron levels have been correlated with poorer neurological outcomes in infants, and lipid peroxidation tends to be higher in those infants who are severely asphyxiated.[30,31]

There are different effects of hypoxia and reoxygenation (H-R) on ROS production in different organs. The liver and myocardium produce high levels of hypoxanthine during hypoxia and skeletal muscle releases large amounts during reoxygenation.[32] The metabolism of hypoxanthine by xanthine oxidase (XO) produces superoxide anion, an ROS.[33]

Antioxidants may attenuate H-R effects. In newborn lambs with severe hypoxic ischemic encephalopathy, antioxidants such as allopurinol (an XO inhibitor), or desferal (an iron chelator), or anti-inflammatory agents improve cerebral oxygen metabolism, carotid blood flow and electrocortical brain activity,[34] with a beneficial effect on free radical formation.[35] Antioxidants can also improve cardiac function on reoxygenation,[36,37] and preserve Na^+-K^+-ATPase activity in cortical cells.[36,38] Nitric oxide (NO) production can lead to reactive nitrogen species release. Post-hypoxic inhibition of NO synthase reduces both lipid peroxidation, and the level of non-protein bound iron.[39] The oxygen concentration used in resuscitation may also affect free radical production and oxygen toxicity may be significant in several clinical scenarios,[40] thus avoiding excessive oxygen delivery may be beneficial.

The current recommendation of the neonatal resuscitation program is to use 100% oxygen when resuscitating asphyxiated infants,[41] although several studies have already shown the equivalence of 21% oxygen with 100% oxygen in attaining good results in resuscitation of hypoxic animal models and infants.[42,43] In newborn piglets, regional and cerebral blood flows, cerebral evoked potentials[44] and pulmonary hypertension,[45] recover equally well. Extracellular cerebral hypoxanthine is lower with 21% resuscitation.[46] Reoxygenation of piglets with meconium aspiration also shows equivalence of 21% and 100% oxygen.[47] Asphyxiated newborn infants have recovered as well, or better, when resuscitated with 21% oxygen compared to 100%. The recovery parameters included acute clinical parameters such as 'time to first breath' and Apgar score, and long-term outcome.[42,48] The oxidative stress of reoxygenation, as measured by the reduced

glutathione:oxidized glutathione ratio (GSH:GSSG ratio), SOD, and catalase activity, is reduced at 3 and 28 days of life, in infants by using 21% oxygen rather than 100% oxygen.[42,49] Oxygen concentrations less than 21% may even be as good. Resuscitation with 18% oxygen is as good as 21% in terms of clinical parameters, and acid base balance in an animal model while reducing to 15% oxygen gives a slower recovery of acid base balance.[50] Parameters of oxidative stress were not investigated in these hypoxic resuscitations, so the role of free radicals is not known. It is worth noting that aircraft flight can reduce oxygen to levels equivalent to 15% at sea level with no apparent harm to infants or adults.[51]

While there has been no long-term clinical disadvantage of 21% oxygen proven, there has also been no long-term advantage identified. This is despite the improvements in oxidative stress shown, and equivalent or better improvement of physiologic parameters in early recovery. It is likely that some infants will recover well whatever type of resuscitation is used as they still have reserves available for buffering of free radicals and production of sufficient ATP. Others will be so severely damaged that the type of resuscitation is immaterial. Some may be close to a threshold where the level of hypoxic injury is such that they will not tolerate the further oxidative stress of resuscitation. These infants are most likely to benefit from optimization of resuscitation, and further consideration of the effect of oxygen levels is needed.

This review will discuss the general mechanisms of cell injury in H-R or ischemia-reperfusion (I-R). The responses in the liver are similar with both types of insult.[52,53] The chemistry and actions of reactive species and their links to the processes

of apoptosis, necrosis and inflammation, and the role of oxygen in the mechanisms will be highlighted. The relevance to hepatic injury in the newborn will also be discussed.

2. Chemistry and actions of reactive species and antioxidants

i) Reactive species

A role for reactive species (ROS, reactive nitrogen species and other free radicals) in clinical asphyxial injury, has been noted above. Among the many biochemical markers of cell damage and cell death, reactive species seem to play a major role as both an indicator and a cause of cellular physiologic stress.

Reactive species are either free radicals or participate in reactions leading to free radical production. Free radicals have an odd number of electrons and take or give electrons to achieve a stable state.[26] The most important reactive species are the ROS (superoxide anion - $O_2^{\bullet}$, the hydroxyl radical - $^{\bullet}OH$ and hydrogen peroxide -H_2O_2) and transition metals (Fe^{2+} and Cu^{+}). Hydrogen peroxide is produced from the dismutation of superoxide anion by SOD. It then reacts with transition metals to produce the hydroxyl radical (the Haber-Weiss reaction [1]).[54]

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+} \qquad [1]$$

Transition metals may also undergo auto-oxidation in the Fenton reaction [2], to produce superoxide anion:

$$Fe^{2+} + O_2 \rightarrow O_2^{\bullet} + Fe^{3+} \qquad [2]$$

Superoxide anion is also produced, by the action of XO, in the mitochondrial electron transport chain [3] and by NADPH oxidase [4].[55]

$$\text{Hypoxanthine (xanthine)} + O_2 + e^- \xrightarrow{XO} \text{Xanthine (urate)} + O_2^{\bullet} + H_2O_2 \qquad [3]$$

$$2O_2 + \text{NADPH} \xrightarrow{\text{NADPH Oxidase}} 2\,O_2^{\bullet} + \text{NADP}^+ + H^+ \qquad [4]$$

Both hydrogen peroxide and the superoxide anion may not be reactive enough to cause cell damage, but they produce the very reactive hydroxyl radical [1]. The hydroxyl radical peroxidizes lipids, proteins and polysaccharides (-R) [5]; and damages DNA.

$$^{\bullet}OH + \text{-RH} \rightarrow H_2O + \text{-R}^{\bullet} \qquad [5]$$

The radical (-R$^{\bullet}$) reacts with oxygen to form the peroxyl radical (-ROO$^{\bullet}$) which can then generate alkoxyl radicals (-RO$^{\bullet}$). Thiyl radicals can also be produced (RS$^{\bullet}$).

The most damaging reaction of free radicals in the cell is lipid peroxidation, particularly of membrane based polyunsaturated fatty acids. A fatty acid radical will form a peroxyl radical, which oxidizes further lipids in an ongoing chain reaction. Transition metal ions are often involved. Newborn infants are particularly prone to oxidative damage from free iron, due to their higher intra-erythrocyte levels.[26,56]

Various aldehydes, particularly hydroxy-alkenals can also be generated from ROS and they diffuse intracellularly to cause further injury. They do not cause damaging chain reactions comparable to those occurring with lipid peroxidation. Protein reactions are local and amino acids can sequester metal ions so these peroxidation reactions may be important in antioxidant defence.[57]

Reactive oxygen species cause further injury by acting as second messengers. They are able to activate transcription factors, which can stimulate cell growth or initiate inflammation or necrosis.[58,59]

Reactive nitrogen species levels are also changed in H-R. Endothelial cells can produce NO. Nitric oxide and superoxide anion radicals produce peroxynitrite ($ONOO^-$), a diffusion limited reaction. Peroxynitrite then decomposes to give rise to hydroxyl radicals [6].

$$3ONOO^- + 3H^+ \rightarrow 3ONOOH \rightarrow {}^{\bullet}OH + {}^{\bullet}NO_2 + 2NO_3^- + 2H^+ \qquad [6]$$

Increased peroxynitrite levels lead to cellular apoptosis, higher levels lead to necrosis probably secondary to oxidant damage.[60] Other reactive metabolites of NO include nitrogen dioxide and nitryl chloride.[26]

The toxicity of radicals is based on a hierarchy (Table 1-1). The radicals can either be more reducing (donate electrons) or more oxidizing (accept electrons). A radical couple with a high reduction potential will accept electrons from a radical with a low potential. Thus depending on the acceptor and donor potentials, radicals may both reduce

and oxidize. The most oxidizing radicals at the top will accept electrons from radicals below them. Within an organism it is the oxidizing radicals that are most deleterious.

ii) Antioxidant strategies

Antioxidant strategies may prevent the formation or release of free radicals or neutralize those already generated. (Tables 1-2 and 1-3)

Morphologically, electron transfer is tightly controlled by the proximity of proteins to each other within the membranes of mitochondria and endoplasmic reticulum to prevent the release of radicals. Transition metal ions are usually bound to proteins, e.g. iron is bound to ferritin to control their reactivity.

Enzymes such as catalase and glutathione peroxidase remove hydrogen and lipid peroxides preventing the Haber-Weiss reaction [1], and the formation of hydroxyl radicals. Superoxide dismutase converts superoxide anion into hydrogen peroxide [7].

$$O_2^{\bullet-} + 2H_2O \xrightarrow{SOD} H_2O_2 + e^- \qquad [7]$$

The non-enzymatic mechanisms neutralize very reactive radical couples to less reactive ones. α-Tocopherol (Vitamin E) breaks the chain reaction of lipid peroxidation.[61,62] The α-tocopherol radical is much less reactive. Ascorbic acid (Vitamin C), a plasma and intracellular antioxidant, neutralizes the α-tocopherol radical. The ascorbate radical is even less reactive and is water soluble, thus removing radicals from the vulnerable lipid membrane. The Vitamin E:Vitamin C ratio is important in maintaining this process.[63] The

absolute levels of ascorbate may also be important. Ascorbate is usually an antioxidant, but it can oxidize metals such as copper and iron due to its position in the hierarchy. It tends to do this when at low concentrations.[64]

Intracellular glutathione is significant as one of the most plentiful intracellular antioxidants, particularly in the liver although its role in liver H-R is not certain.[65] It neutralizes such oxidants as hydrogen peroxide and peroxynitrite [8], [9], [10].

$$2GSH + H_2O_2 \xrightarrow[\text{Peroxidase}]{\text{GSH}} GSSG + 2H_2O \qquad [8]$$

$$2GSH + ONOO^- \longrightarrow GSSG + 2H^+ \qquad [9]$$

$$GSH + ONOO^- \longrightarrow GSNO + H^+ \qquad [10]$$

Uric acid plays an antioxidant role in plasma.[54,66] Some defences against radicals such as ferritin merely sequester the active molecules without reacting with them.

Redox couple	$E^{0'}$/mV
$OH^{\bullet}, H^{+}/H_2O$	+2310
$RO^{\bullet}, H^{+}$/ROH (aliphatic alkoxyl radical)	+1600
$ROO^{\bullet}, H^{+}$/ROOH (alkyl peroxyl radical)	+1000
$GS^{\bullet}$/GS (Glutathione)	+920
$PUFA^{\bullet}, H^{+}$/PUFA-H (polyunsaturated fatty acids)	+600
$HU^{\bullet-}, H^{+}/UH_2^{-}$ (Urate)	+590
$TO^{\bullet}, H^{+}$/TOH (Tocopherol)	+480
$H_2O_2, H^{+}/H_2O, HO^{\bullet}$	+320
$Ascorbate^{\bullet-}, H^{+}$/ Ascorbate monoanion	+282
Fe(III)EDTA/Fe(II)EDTA	+120
$O_2/O_2^{\bullet-}$	-330
Fe(III)DFO/Fe(II)DFO (Desferal)	-450
$RSSR/RSSR^{\bullet+}$ (GSH)	-1500
H_2O/e^{-}	-2870

Table 1-1 Reduction potentials at pH 7 for radical couples. [63]

Antioxidant	Notes
Cytochrome oxidase	Accepts 95-99% of cellular O_2
Superoxide dismutase	Dismutates $O_2^{\bullet}$ to H_2O_2
Catalase	Detoxifies H_2O_2
Glutathione Peroxidase	
Peroxidase	

Table 1-2 Enzymatic endogenous protection from radicals (Modified from Rangan 1993,[66] Buttke 1994,[115] Carmody 2001.[59]

Antioxidant	Notes
Lipid soluble	
α-tocopherol (Vit E)	$LOO^{\bullet}$ scavenger, stops chain reaction
β-carotene (Vit A precursor)	Stops chain reactions
Water Soluble	
Glutathione	Most ubiquitous intracellular antioxidant
Thioredoxin	
Ascorbic acid (Vit C)	Neutralizes Vit E
Uric acid	$O_2^{\cdot}$ and $OH^{\bullet}$ scavengers
Cysteine	
Bilirubin	
Albumin	$LOO^{\bullet}$ and HOCL scavenger
Caeruloplasmin	
Transferrin	Bind circulating iron
Lactoferrin	
Ferritin	Binds tissue iron
Amino acids/proteins	Non specific buffering
Bcl-2	GSH levels increased
	GSH translocation to nucleus increased

Table 1-3 Non-enzymatic endogenous protection from radicals (Modified from Rangan 1993,[66] Buttke 1994,[115] Carmody 2001.[59]

3. Cellular effects of hypoxia and reoxygenation

i) Reactive species production and activity

The various physiologic sources of reactive species in a cell may be activated by hypoxia and potentiated by reoxygenation. The amount of oxygen available to support these reactions and the availability of antioxidants are two important factors in determining injury in H-R.

a) In hypoxia

Hypoxia reduces antioxidant resources. With increasing periods of hypoxia, there is progressive down regulation of hepatic GSH levels and glutathione peroxidase levels,[67] and also decreased SOD, catalase and cytochrome oxidase activity.[5] Hypoxia and anoxia are also associated with increased ROS production. Production sites include the cytosol and mitochondria. Calcium builds up in the cytosol in hypoxia due to a lack of ATP, and dysfunction of the Na^+-K^+-ATPase and the Ca^{2+}-ATPase.[5] The Ca^{2+} activates phospholipase A_2 and C, producing free fatty acids and lipid free radicals from the peroxidation of polyunsaturated fatty acids as described above.[26,54] Mitochondria produce superoxide anion as an integral part of the electron transport chain [3].[68] Excessive superoxide anion can be produced in hypoxia. When the terminal processes involving cytochrome oxidase in the electron transport chain are inhibited by the lack of oxygen, and the chain is fully reduced, there is overspill of excess superoxide anion.[69] High cytosolic Ca^{2+} in hypoxia can lead to abnormal Ca^{2+} cycling in the mitochondria

and also increases ROS production.[70] Tumor necrosis factor -α (TNF-α), produced in hypoxia, also induces cytocidal mitochondrial ROS production.[71] Concomitant disorganization of the inner mitochondrial membrane disrupts the electron transport chain and the tight control of radical transfer with further release of ROS. Mitochondrial death and rupture can also release the ROS into the cytosol.[26]

b) In reoxygenation

Reactive species are also produced in reoxygenation or reperfusion. They are produced in several cellular locations by stimulation of metabolic pathways and also as part of an inflammatory response. Responses may be organ specific. There is a biphasic response in the liver with a sharp increase at the onset of reperfusion, a subsequent decrease, then a steady rise commencing at 30-60 minutes after reperfusion,[63] whereas in the myocardium the highest levels of free radicals are seen in the first 10 minutes of reperfusion.[69] The cellular locations include the cytosol, mitochondria, membrane bound enzymes and microsomes.

Cytosolic xanthine dehydrogenase (XDH) is converted to XO during hypoxia, regulated by Ca^{2+} dependent calpain.[72]. On reperfusion XO breaks down the large amount of hypoxanthine and xanthine, accumulated in hypoxia and produces superoxide anion. The mitochondria also produce excess superoxide anion, with very high oxygen levels.[69] Other sources of ROS are cyclooxygenases,[73] lipoxygenases[74] and NADPH-oxidase.[73,75] NADPH oxidase is present intracellularly in some parenchymal cell microsomes, and is

particularly important for the oxidative burst of neutrophils.[76] Endothelial cells express a membrane bound NADPH oxidase and can release extracellular superoxide anion.[77]

Certain mediators have been implicated in initiating the production of reactive species. Tumor necrosis factor -α, probably from Kupffer cells,[78] is produced between 15 minutes and 4 hours after the reperfusion of ischemic liver.[24 79] It induces ROS production in both the cytosol and mitochondria, at a later time than the initial rise in ROS on reoxygenation. It seems to accelerate the physiologic mitochondrial ROS production.[71] The prevention by antioxidants of the TNF-α induced stacking of mitochondrial cristae and formation of multilamellar structures, also links reactive species to TNF-α activity.[80] Tumor necrosis factor -α and hypoxia also activate inducible NO synthase in respiratory endothelial cells,[81] and hepatocytes at a transriptional level,[77] which can give rise to NO and peroxynitrite in the presence of superoxide anion.

Inflammatory reactions also produce ROS. Kupffer cells are activated in the first 2 hours of reperfusion,[24] and release superoxide anion and hydrogen peroxide, stimulated in part by complement. Removal of Kupffer cells from transplanted organs prevents reperfusion injury.[82] Interleukins and TNF-α mediate later inflammatory changes. For example increased pulmonary permeability occurs after 9-12 hours of reperfusion, 5-8 hours after the TNF-α peak.[24] Tumor necrosis factor -α also causes increased endothelial cell adhesion molecule production with increased platelet and neutrophil attachment, and translocation of the NADPH oxidase complex from the cytoplasm to the cell membrane, particularly in monocytes and neutrophils.[6,78] Inhibition of TNF-α with antiserum reduces neutrophil infiltration, and the reduction of neutrophil ROS release may mediate a

concomitant reduction of liver injury.[79] Interleukin-1 causes Kupffer cells to release free radicals and TNF-α. Its inhibition reduces neutrophil infiltration and hepatocyte damage.[83,84] Interleukin-12 has a similar effect.[85]

There are several other potential intracellular and extracellular stimulators of reactive species production in H-R. Platelet activating factor, produced by the liver rises during the first 12 hours of reperfusion. Its inhibition reduces lipid peroxidation and improves the survival of transplanted liver.[83] Low serum pH during hypoxia causes iron release from transferrin, which reacts to produce free radicals, as previously described.[86,87] These free radicals can cause further iron release from ferritin, setting up a vicious cycle. Calpains are expressed in the liver during ischemia and reperfusion in vivo.[88] Calpains are non-lysosomal, Ca^{2+} dependent, membrane based proteases that lyse cytoskeletal and membrane based proteins. They increase XO production, play a role in neutrophil exocytosis and activate phospholipase C, all of which can increase free radical production. Calpain inhibition seems to reduce both signs of necrosis and morphological apoptosis.[89]

There are therefore multiple pathways for injurious reactive species to be produced in both hypoxia and reoxygenation, both intracellularly in the cytosol, microsomes and mitochondria and extracellularly, particularly from membrane bound NADPH oxidase.

ii) Cell death and reactive species

Cell death is important in the development of organ dysfunction in H-R injury. Reactive species have been implicated in the different modes of cell death. There are two recognized modes of cell death, apoptosis (energy dependent programmed cell death) and necrosis. Often, there is overlapping of the two modes, and mitochondria play a significant role in both. With hypoxia or anoxia, ATP production is reduced,[90] and this serves initially to preserve the inner mitochondrial membrane potential. However, this also leads to ATP depletion and a build up of ROS[91,68] and both can be cell damaging. When the mitochondrial membrane potential is eventually lost (the mitochondrial membrane permeability transition - MPT), there is a leakage of proteins into and out of the mitochondria causing mitochondrial swelling and death, with possible subsequent cell necrosis or induction of apoptotic pathways.[92] The MPT occurs after 30-60 minutes of hypoxia in hepatocytes.[91] Apoptosis will progress if reoxygenation occurs with resumption of ATP production from still viable mitochondria.

Apoptosis morphology is similar between cell types.[6,93] There is membrane blebbing, cell shrinkage and nuclear condensation with breakdown of DNA. In vitro the cells come apart, as well formed apoptotic bodies. Apoptosis can be induced in H-R by endogenous and exogenous mechanisms. There are 3 phases to the process: the *induction* phase (death inducing receptors and intracellular proteins are activated or translocated), the *effector* phase (proteases are activated), and the *degradation* phase (morphological and biochemical markers of apoptosis are apparent).[94]

The exogenous mechanism of apoptosis involves activation of a death receptor by a ligand. Tumor necrosis factor-α, from the systemic circulation, or from a paracrine or autocrine source, seems to be the main ligand in H-R injury.[6] Tumor necrosis factor -α produced during the reoxygenation phase,[79] activates caspase-8 in parenchymal cells,[73,95] which activates effector caspases. Caspases are a family of cysteine proteases involved in the initiation and the effector phases of apoptosis,[96] resulting in a lethal protease cascade.[97] Caspase-8 can also initiate apoptosis by causing Bax or Bak protein translocation into mitochondria causing cytochrome c release,[98] which activates downstream caspases.[99] Bax and Bak are members of the Bcl-2 family of proteins,[100] which are involved in apoptosis either by promoting or inhibiting it.

In the endogenous pathway, ATP depletion, increased Ca^{2+} levels[101] in the mitochondria, and spontaneous Bax or Bak translocation to the mitochondria during hypoxia cause the MPT. Cytochrome c is released into the cytosol and combines with apaf-1 using ATP to form the apoptosome and activate caspases.[5,102] Other proteins such as smac[103], which inactivates Inhibitor of apoptosis proteins (IAP), are also released from the mitochondria.[97,102] The Inhibitor of apoptosis proteins constitutionally bind caspases-3 and -7 to keep them inactive.[104]

Redox stress also mediates apoptosis via calpain mediated release of caspase 12 from endoplasmic reticulum.[88] Calpain activation in ischemia is related to the duration of ischemia and there is a continuing rise for at least 2 hours after reperfusion.[88] In some models calpain inhibition completely prevents apoptosis in liver reperfusion injury.[105]

Cells also have other intrinsic apoptosis inhibitors. Bcl-2 and Bcl-X_L inhibit the MPT.[6,94,106] Bcl-2 may also block lipid peroxidation by increasing the cellular uptake of ascorbic acid and it also inhibits caspase-3 activation.[107,108] Heat shock proteins produced by oxidant stress are also protective.[109-111]

The degradation phase ensues once the effector caspases or similar proteases are activated, with the characteristic morphological features described above.[6] Blockade of the apoptosis cascade in vitro can prevent cell death but only if the cell is not completely depleted of ATP and there remain sufficient viable mitochondria to avoid necrosis.[112] In vivo or clinical applications of such inhibition have not yet been achieved in H-R.

Numerous studies have identified a role for reactive species in apoptosis. An increased partial pressure of oxygen may enhance apoptosis. In hematopoetic cells it increases hydrogen peroxide levels and the effect can be attenuated with antioxidants.[113] The addition of hydrogen peroxide, in low doses, to tumor cells causes apoptosis, with higher doses causing necrotic pathology.[114] In some T-cell lines, higher levels of extracellular catalase protect cells from hydrogen peroxide induced apoptosis.[115] A pathway involving hydrogen peroxide and TNF-α activates the apoptosis signal-regulating kinase 1, inducing apoptosis in fibroblasts.[116] The activation of NADPH oxidase in neurons produces superoxide anion with associated apoptosis,[117] and a homologous oxidase in many cell types may produce superoxide anion when activated by the GTPase, Rac 1.[118] Rac-1 regulated ROS production also leads to apoptosis.[119]

The effect of antioxidants also supports a role for reactive species in apoptosis. The TNF-α mediated death of lymphoma cells is associated with a rise in ROS, can be

inhibited by thioredoxin,[120] and the sensitivity to cell death correlates with SOD levels.[121] The number of apoptotic cells in myocardium also seems to be reduced on reperfusion, if there is preperfusion of SOD and catalase.[122] Bcl-2 as well as inhibiting apoptosis at a mitochondrial level may act as a more general antioxidant. It attenuates oxidant stress by interacting with thiols.[123] It can also cause relocation of GSH to the nucleus and increased cellular GSH levels.[124] Transcription factors such as p53, AP-1 and NF-κB, which are involved in cell death can be activated by changes in redox potential within the nucleus.[125]

Thus the presence of reactive species and the protective effect of antioxidants both support a causative role for the reactive species in apoptosis. It is not clear in many of the studies how the reactive species affect the known pathways of apoptosis, nor is it clear how oxygen levels affect their activity.

Necrosis is the end result of various malfunctions in cellular function and is thus less clearly defined than apoptosis, in terms of specific metabolic pathways. Morphologically, necrotic cells swell up and then collapse with release of their contents into the surrounding medium, and the features of apoptosis are absent.[6] Damage is known to occur due to many subcellular dysfunctions. Prolonged hypoxia may lead to irreparable mitochondrial damage and cell death by necrosis even before reoxygenation.[126,127] Dysfunctional Na^+-K^+-ATPase in hypoxia allows Na^+ and Cl^- into the cytosol, and the endoplasmic reticulum similarly releases Ca^{2+} due to a lack of ATP. High intracellular Ca^{2+} levels activate phospholipase A_2 and C with membrane lipid

hydrolysis and free radical production. This effect of Ca^{2+} is concentration dependent and preservation of glycine levels may alleviate the effect.[101]

Thus the processes leading to necrosis in H-R are linked to ATP depletion, intracellular electrolyte disturbances, dysfunction of homeostatic mechanisms and free radical activity.

Thus although cell death certainly occurs due to ATP depletion with prolonged hypoxia, reactive species also play a role and particularly during reoxygenation or reperfusion.

4. Specific hepatic responses to hypoxia-reoxygenation

The various types of cell in the liver respond differently to H-R although reactive species can be produced by, or affect most of them. On reperfusion there is a biphasic reactive species release in the liver with a sharp increase at the onset of reperfusion, a subsequent decrease, and then a steady rise commencing at 30-60 minutes after reperfusion.[65] Antioxidants such as SOD[128] or exogenous hydrophobic antioxidants,[71] can inhibit the hepatic necrosis consequent on the later TNF-α induced reactive species production.[71]

i) Cellular responses

Some specific hepatic effects of reactive species and cell death have been considered above. However, the liver contains several different cells: Kupffer cells, sinusoid endothelial cells (SEC), hepatocytes, biliary epithelial cells and hepatic stellate cells. The various cells have different susceptibilities; for example hepatocytes are more vulnerable to anoxia than endothelial cells, whereas they are less vulnerable to the oxidative stress of reoxygenation due to their abundant cytosolic antioxidant capacity.[91] Cellular interactions are also important. The viability of reoxygenated hepatocytes in a reperfused ischemic liver is less than that of hepatocytes extracted from the same organ during ischemia, and reoxygenated in cell culture.[129] The hepatocyte seems resistant to pure hypoxia or hyperoxia and but is vulnerable to injury when interacting with other cells and circulating components.

Kupffer cells in the liver are the largest concentration of fixed macrophages in

any organ, so can produce much TNF-α,[24] and also generate free radicals.[26] Removal of the Kupffer cells attenuates liver sinusoidal cell injury by about 50% and blocking Kupffer cell activity with gadolinium chloride equally reduces hepatocyte damage.[77] Co-culturing hepatocytes and Kupffer cells leads to greater reoxygenation damage to hepatocytes than if the hepatocytes are isolated.[130] It is not known if these effects are due to removal of TNF-α, ROS production, cytokine production or a combination.[82,83] Activating Kupffer cells causes oxidative stress and leads to raised GSSG levels.[131] The Kupffer cell is therefore a significant hepatic cell in I-R. Residual damage still occurs even with removal of Kupffer cells and sinusoidal cells are particularly affected.

Hepatic SEC are active in the reperfusion/reoxygenation injury process. They detach from the basement membrane during ischemia and if their integrin receptors are not blocked they undergo apoptosis on reperfusion.[105] Reactive species can be both produced by and affect the SEC. In vitro, SEC undergo cell death in the reoxygenation phase of H-R and exogenous GSH, catalase and desferrioxamine all have a protective effect.[132] Apoptosis is also induced in SEC by hydrogen peroxide from hepatocytes, which does not damage the hepatocytes.[133] Much or all of this hydrogen peroxide is derived from the hepatocyte mitochondria.[134,135]

Isolated hepatic SEC have been noted to contain cytosolic XO.[136] However, the isolated hepatocyte does not seem to produce excess superoxide anion from this source.[76] In intact liver as opposed to cell culture, the peak of XO activity is 1 minute after reperfusion and the activity then falls rapidly to pre-hypoxic levels.[52] This may account for at least some of the initial part of the biphasic pattern of ROS production. The

significance of reactive species production from XO is uncertain, as allopurinol (an XO inhibitor) does not reduce the oxidative stress in the liver in some animal models,[137,138] and GSSG levels can be unchanged.[65]

Endothelial cells also express a membrane NADPH oxidase and can release superoxide anion.[77] They also produce NO,[139] as do hepatocytes and inflammatory cells, leading to reactive nitrogen species release.[83,140] Sinusoidal endothelial cells also produce matrix metalloproteinase-2 in H-R causing SEC apoptosis.[139] In the brain and heart, 100% oxygen compared to 21% oxygen resuscitation, will increase matrix metalloproteinase-2 production post hypoxia, suggesting a role for ROS in its production,[141,142] but similar results have not yet been shown for SEC.

Sinusoidal endothelial cells are also involved in neutrophil accumulation by interactions via adhesion molecules and chemokine release. The expression of adhesion molecules is induced by TNF-α and reperfusion injury is reduced by antibodies against them.[78] Depletion of circulating leukocytes reduces lipid peroxidation,[143] demonstrating the synergy of SEC and leukocytes in causing ROS induced cellular injury.

One effect of the ROS on endothelial cells is to activate redox sensitive transcription factors such as NF-κB which control the endothelial adhesion molecules. They also activate phospholipase A_2, which leads to platelet activating factor production. Platelet activating factor stimulates leukocyte and platelet and endothelial interactions.[55] Platelets are also known to be activated independently by an ROS dependent process in H-R,[144] and they cause reduced hepatic glutathione levels.[143] Platelet adhesion causes endothelial cell apoptosis, without damaging hepatocytes. If their adhesion is prevented,

this effect is significantly reduced.[145] These blood component-endothelial interactions are markedly attenuated by a variety of antioxidants, emphasizing the pivotal ROS role.[55]

The SEC, like the Kupffer cell, thus seems a pivotal cell in H-R due to its susceptibilty to H-R injury and its reactions with other cells. Reactive species play an important although not exclusive role in these processes.

Hepatocytes are more resistant to oxidative stress This may be due to their high content of antioxidants such as glutathione, which are well maintained in hypoxia and reoxygenation, relative to other organs.[8] It could also be due to the maintenance of Bcl-2 levels in hepatocytes during hypoxia, while the levels fall in SEC secondary to hepatocyte hydrogen peroxide production.[146] The presence of heat shock proteins in both hepatocytes and SEC protects cells by inhibiting downstream apoptosis. Heat shock protein production can be induced by ischemic pre-conditioning,[147,148] and the level of mRNA production correlates with the hepatic oxygen debt.[149] Heat shock protein production in hepatocytes is associated with an increase in hydrogen peroxide production in adjacent hepatocytes. The inhibition of ROS with beta-carotene and allopurinol during reoxygenation can ablate this increase in heat shock proteins.[150] Hence, reactive species are not exclusively deleterious and may stimulate cellular defences while antioxidants can impair them. Hepatocyte capacity for resistance to injury can vary between species with rat hepatocytes being less resistant than human ones to anoxia and reoxygenation.[151] This highlights the need for caution in applying animal model results directly to clinical situations.

Hepatocytes also produce reactive species. Mitochondria are probably the main source of ROS in reperfused liver, since SOD but not catalase or allopurinol, attenuate much of the ROS production.[138] NADPH oxidase is present in hepatocyte microsomes,[76] and its absence in a knockout model greatly reduces hepatic necrosis after hemorrhage and resuscitation. There is also an associated reduction in neutrophil infiltration, lipid peroxidation and nitrotyrosine levels.[75] Reactive oxygen species are also produced in hepatic microsomes by cytochrome P_{450} oxidases on reoxygenation.[77]

The biliary epithelial cells, like the SEC, are more resistant to anoxia, but less so to reperfusion than hepatocytes. Less is known of the specific cellular response to H-R injury in these cells, although biliary strictures have been described[91] and cholestasis has been noted in asphyxiated infants.[20]

In hepatic stellate cell cultures, hypoxia induces matrix metalloproteinase-2 activity and mRNA expression, and inhibits the tissue inhibitor of matrix metalloproteinase-2,[152] while hyperoxia causes a rise in both. Hepatic stellate cells are the main fibrogenic cells in the liver[153] and as such, they are more significant in long term fibrotic diseases than acute H-R. Despite the response to hyperoxia, it is not known if reactive species are involved in the pathway.

Other studies have also shown links between reactive species and hepatic I-R or H-R injury, without identifying the cell types involved.

Necrosis of cells, as indicated by serum lactate dehydrogenase-5 levels, correlates with levels of aldehyde modified proteins,[154] and there is a linear relationship between irreversible cell damage and redox imbalance after liver transplantation.[155]

The buffering of reactive species reduces injury. Inhibition of XO with allopurinol prior to reperfusion improves the return to baseline of hepatic blood flow,[156] although other studies suggest there is no role for XO in hepatic I-R injury.[157] Administration of melatonin reduces hepatic lipid peroxidation and neutrophil infiltration, while preserving GSH.[158] Scavenging of hydroxyl radicals in vivo reduces lipid peroxidation, improves systemic and hepatic hemodynamics and energy metabolism and improves survival in animal models.[159] Maintaining glutathione levels with precursors such as N-acetylcysteine,[160] or γ-glutamylcysteine ethyl ester,[161] reduces lipid peroxidation and enhances recovery of mitochondrial function.

Reducing antioxidants is deleterious. Inducing glutathione deficiency enhances the detrimental hepatocellular fluid uptake, which occurs during reperfusion, while allowing higher malondialdehyde accumulation and higher serum transaminases.[162] Glutathione deficiency also decreases the buffering of Ca^{2+}-mediated ROS production.[163]

ii) Hemodynamic responses

In I-R experiments the perfusion of an organ is controlled. In H-R experiments, ischemia occurs secondary to hypoxia and there are well described differential organ effects.[164,165] Several studies have identified hepatic changes:

Hepatic blood flow in neonatal lambs is maintained until the arterial plasma O_2 content falls below a critical threshold of 6.5 ml O_2/dl. The fall is mostly due to reduced portal vein flow. Above the threshold oxygen consumption is maintained, despite lower

oxygen delivery, due to increased oxygen extraction. Once flow deteriorates increased extraction cannot compensate. Oxygen consumption by the neonatal lamb liver is thus mostly flow dependent.[166]

In an almost ideal model of perinatal hypoxia, Bristow et al[167] investigated the hepatic responses to systemic hypoxia in a fetal lambs. They showed a reduction in hepatic blood flow of 20% with the right lobe flow falling more than the left. This is probably related to flow characteristics through the umbilical vein and ductus venosus. Hepatic arterial resistance is increased in dogs during severe hypoxia, but not in moderate hypoxia.[168] The dual blood supply thus keeps the hepatic flow stable until a critical threshold by means of the hepatic arterial buffer response. The hepatic artery can increase flow to compensate for portal vein variability due to a local effect of adenosine.[169,170]

Cheung et al[164] have demonstrated rapid recovery of total hepatic flow on reoxygenation, but not later responses. Vasoactive substances when present, can impair sinusoidal flow during reperfusion. Endothelin causes sinusoid constriction and hepatocellular injury.[171-173] The no-reflow phenomenon occurring later in reperfusion thus may be related to endothelin which is associated with ROS production.[174]

iii) Oxygen metabolism

The liver in newborn lambs can maintain oxygen consumption even with reductions in oxygen delivery of up to 50% due to its' ability to increase oxygen extraction up to 81%.[175] A large enough reduction in hepatic blood flow will however lead to a reduction in oxygen consumption.[166] The reduction correlates with reduced

delivery in fetal lambs, and there is a simultaneous release of glucose into the circulation to aid compensatory glycolysis.[167] An intact rat model has shown a reduction in the activity of enzymes associated with energy metabolism, such as cytochrome oxidase, in the liver during ischemia. This could explain the reduction in oxygen consumption observed in the fetal lambs.[176]

In young piglets global hypoxia has also been shown to reduce hepatic perfusion and oxygen consumption in excess of any cerebral effect.[164,165] Early reoxygenation did not immediately improve oxygen consumption, probably due to impaired cardiac function. Hence in an intact animal, cardiac dysfunction may cause an ongoing hepatic insult even during reperfusion.

The neonatal or fetal liver thus maintains oxygen consumption well up to a threshold. Reoxygenation and its effects are not well described.

iv) Downstream organ effects of hepatic hypoxia and reoxygenation

It is well recognized that the effluent from a reperfused liver will cause acute lung injury with inflammation and lung chemokine production,[177,178] and also adverse pulmonary hemodynamics.[179] Reactive species involvement is shown by perfusing the lung with N-acetyl-cysteine during initial reperfusion. This replenishes glutathione reserves and prevents pulmonary edema and hypertension.[180] Tumor necrosis factor -α from the liver is an important initiator of lung effects,[79] and causes edema up to 12 hours post reperfusion.[24] Inhibiting the inflammatory cascade with interleukin-10 will reduce

lung inflammation,[181] although the effect on pulmonary hemodynamics is not known. Thus, there seems to be an early hemodynamic response preventable with antioxidants and a later inflammatory response where the role of antioxidants is less clear.

5) Oxygen levels: cell and organ effects

During reoxygenation, reduced partial pressures of oxygen could be beneficial in decreasing reactive species production. Increased oxygen levels might be expected to cause more reactive species induced damage, and some studies seem to confirm this: The toxicity of oxygen is seen in hematopoetic cells when an increased partial pressure of oxygen enhances apoptosis initially induced by other mechanisms. The cause is increased hydrogen peroxide levels, and the effect can be attenuated with antioxidants.[113] Prolonged hyperoxia of 24 hours has also been shown to be detrimental in immature rat brains due to peroxynitrite production.[182] The effect of short periods of hyperoxia is less well defined, particularly in the context of H-R, although greater oxidative stress occurs if higher levels of oxygen are used during resuscitation of infants.[49]

The interaction of hypoxia and reoxygenation is complex and variable, it is not merely the hyperoxia that produces the ROS, the hypoxia also has a role.

In the liver the duration of hypoxia or ischemia is positively correlated with subsequent reactive species release.[183] Hypoxia with low hepatic blood flow can be more detrimental than total anoxia, as cells are subject to both ATP depletion and exposed to sufficient oxygen to allow reactive species production.[91] If a shorter period of hypoxia is interrupted by a brief reoxygenation prior to further hypoxia, hepatocellular apoptosis occurs after less total hypoxia, than if the organ had experienced the hypoxia as one long period.[184] In contrast a period of hypoxia/ischemia with a long recovery time (ischemic preconditioning) can reduce the degree of injury.[185] However, hypoxia will not cause the

ultrastructural changes in mitochondria due to H-R such as swelling,[186,187] reoxygenation seems to be necessary.

Giving oxygen in excess of requirements does seem to be detrimental to oxygen consumption in some critically ill adults.[188] However, in specific situations, such as hypothermia, it has been shown in newborn rats that hyperoxia can be beneficial in maintaining metabolism.[189] In normal, as opposed to post-hypoxic newborn humans, hyperoxia can also allow increased oxygen consumption.[190] The definition of excessive oxygen is therefore relative to the needs of the newborn.

In perinatal asphyxia the hypoxia is not the phase of the illness that can be treated. However, understanding the mechanisms of injury, in both the hypoxic and reoxygenation phases and the protective mechanisms that can be induced, will be necessary to refine treatments in the phase that can be influenced, namely the reoxygenation phase. Reducing oxygen levels given to asphyxiated infants is one possible strategy.

6) Summary

Cell death and organ dysfunction occur in the newborn liver subsequent to H-R injury. Hemodynamics and oxygen metabolism can be impaired. Reactive species play an important role in cellular responses and may be important hemodynamically due to effects on SEC. Antioxidants are beneficial in vitro and to some extent in vivo in preventing hepatic H-R injury but are not a panacea. The effect of oxygen levels in the liver during resuscitation is not known. Studies in intact animals modelling perinatal asphyxia have not addressed hepatic effects to a great extent. Investigating hepatic hemodynamics and oxygen metabolism in intact animal H-R and the relation to reactive species may be of benefit in further understanding the pathophysiology of perinatal asphyxia.

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Chapter 2

Development of a newborn piglet model to study hepatic responses to hypoxia and reoxygenation.

Newborn model of hepatic H-R

Background

Perinatal asphyxia causes significant neonatal and childhood morbidity and mortality.[1,2] It is a multisystem disorder, which can involve the liver. The liver may contribute to the pathology in the asphyxiating process as well as being affected by it. For example, high ammonia levels are associated with encephalopathy and are seen in asphyxiated infants,[3,4] and coagulation abnormalities arise from liver dysfunction.[5]

The liver is resistant to hypoxia compared with other organs, as hepatocytes can reversibly reduce their metabolic activity to a marked degree,[6] and also markedly increase oxygen extraction.[7] Decompensation will, however, occur beyond certain thresholds. The newborn's liver is dependent on blood flow and efficient oxygen extraction, being less tolerant of ischemia than hypoxia. The portal system is more important than the hepatic artery in providing this flow. Hypoxia, however, eventually causes secondary ischemia and at this point the liver's oxygen supply becomes compromised.[7,8] The hepatic artery can buffer these reductions in portal flow, but to a limited extent.[9,10]

Hypoxic injury is followed by reoxygenation injury, but the effects of reoxygenation in the newborn's liver, including hemodynamics and oxygen metabolism, have not been fully examined. The brain and the heart have been well studied in this context,[11-14] but outside of surgical procedure or transplant related research,[15,16] little work has been done on the liver. The liver has a significant reserve of antioxidants such as glutathione[17] and liver injury can affect other organs.[18,19,20] Strategies developed to

minimize hepatic injury from ischemia-reperfusion and to maximize recovery during and after liver transplant[16] have not been assessed in hypoxic infants.

Swine have been used extensively as models of human disease,[20] and have proved to be a suitable model for human perinatal conditions.[21] Some aspects of hemodynamic changes and oxygen metabolism of the liver in hypoxia have also been described in piglets.[10,22] We thus, developed an intact newborn piglet model of neonatal asphyxia, designed to more fully examine the hepatic hemodynamics, oxygen metabolism, biochemical markers of tissue injury and histologic features during hypoxia-reoxygenation (H-R).

Methods

The study conformed to the regulations of the Canadian Council of Animal Care (Revised 1993) and was approved by the Health Sciences Animal Welfare Committee, University of Alberta.

Animals

Six piglets were studied, without inducing H-R, to identify the physiological effects of the model. The animals were newborn mixed breed piglets between 1-3 days old and 1.5-2.0 kg in weight. They were obtained from local breeders on the day of experimentation. Their weights were determined and they were observed for 30 min to allow for stabilization.

Surgery

The animals were kept on a radiant warmer (Ohio NC, Neonatal Care Center) for the duration of the experiment. Anesthesia was induced with isoflurane (5%) and oxygen mixture at 3 l/min via a sealed mask for 3 min. Anesthetic gases were delivered by an anesthesia machine (VMS Fraser-Harlater). The isoflurane was reduced to 2% for maintenance anesthesia. The animals continued to breathe spontaneously. With the establishment of venous access, inhalational anesthetics were discontinued. A bolus of fentanyl (10 μg/kg) and of acepromazine (0.1 mg/kg) were given intavenously. Intravenous (IV) infusions of fentanyl at 5-10 μg/kg/h, midazolam at 0.1-0.2 mg/kg/h,

and pancuronium at 0.05-0.1 mg/kg/h were commenced. Further pancuronium (100 μg/kg) or fentanyl (10 μg/kg) boluses were given for muscle paralysis and analgesia, respectively, as required.

Via a groin incision 5 Fr single lumen catheters (Sherwood Medical Co., St Louis, MO) were inserted to 7 cm in the femoral artery and vein and sutured in place. The arterial line was used to monitor blood pressure and for blood sampling. The venous line was used for IV medications and maintenance infusion fluids. A 5 Fr single lumen right external jugular catheter (Sherwood Medical Co.) was positioned for central venous blood sampling and central venous pressure (CVP) measurement.

A 3.0 mm endotracheal tube (Mallinckrodt, St Louis, MO) was inserted using a tracheotomy. With the onset of paralysis assisted ventilation with a neonatal ventilator (Model IV-100, Sechrist Inc., Anaheim, CA) was commenced. Inspired oxygen concentrations (FiO_2) were monitored with an in-circuit oxygen analyzer (Ohmeda S100, Ohmeda Medical, Laurel, MD). Baseline ventilation was pressure controlled at 16/4 cm H_2O, at 12-18 breaths per min with an FiO_2 of 0.21-0.25.

Via a left thoracotomy in the third intercostal space, a 6 mm ultrasonic flow probe (6SB Transonic, Ithaca, NY) was placed around the main pulmonary artery (PA) to measure PA flow.

A laparotomy was then performed. The porta hepatis was dissected and 1 mm and 3 mm ultrasonic flow probes (1SB and 3SB, Transonic) were placed around the main hepatic artery (HA) and portal vein (PV), respectively. All flow probes were zero calibrated prior to use. The umbilical vein was catheterized intraperitoneally with a 5 Fr

catheter (Sherwood Medical Co.), with the tip placed at the junction with the left PV under direct vision. The liver was mobilized with minimal handling by division of the falciform ligament. To gain access to the hepatic vein, the right hemidiaphragm was divided from the anterior costal margin to a point 5 mm anterior to the right hepatic vein. The thorax was opened at the right costal margin to allow an appropriate angle of approach to insert a cannula. An 18 G IV cannula (B-D, Franklin Lakes, NJ) was fenestrated 2 mm behind the tip to optimize blood flow and was inserted into the right hepatic vein and secured. It was flushed with heparinized 0.9% saline after sampling to ensure patency.

The laparotomy and thoracotomy wounds were loosely closed and covered with warm saline soaks to minimize fluid loss. The duration of the surgical procedure was 60-70 min.

A dextrose-saline infusion was maintained at 15 ml/kg/h. Sodium bicarbonate was infused at rate of 0.25 mmol/kg/h in all animals. To avoid hypovolemia, an IV bolus of Ringer's lactate (10 ml/kg) was given at the time of initial paralysis, at jugular line insertion and prior to the laparotomy. Further boluses of 10 ml/kg, were given outside of the stabilization period, if the CVP fell below 5 cm H_2O or if PA flow was <120 ml/min.

Stabilization protocol

The period of post-operative stabilization was 60 min. An initial arterial blood gas was performed to adjust the ventilation to maintain the p_aO_2 at 60-80 mmHg, the p_aCO_2 at 35-45 mmHg, and the pH at 7.35-7.45. Any base deficit of greater than 5 was corrected

at this point with 4.2% sodium bicarbonate. The hemodynamic measurements of flow were required to be stable with less than 10% change during the last 15 min of the stabilization period. The rectal temperature was kept at 38.5-39.5°C.

Experimental protocol

The piglets were observed for a period of 240 min. They were ventilated with 21% oxygen to maintain transcutaneous hemoglobin saturations of >95%. Hemodynamic recordings and blood samples were taken at pre-defined intervals.

After the 240 min period the piglet was euthanized with an IV injection of pentobarbital (240 mg), and the liver tissue harvested for subsequent analysis.

Hemodynamic measurements and recording

Heart rate, via a 3-lead ECG, mean arterial blood pressure (MAP) and CVP, were continuously monitored using a Hewlett Packard cardiomonitor (Model HP 78833B). Transcutaneous arterial oxygen saturation (S_aO_2) was monitored with a pulse oximeter (Nellcor, Hayward, CA). The blood flows at the PA, HA and PV were also continuously monitored throughout the study period. The analogue outputs of these hemodynamic measurements were digitized with a DT2801-A analogue to digital converter (Data translation, Ontario, Canada). Data for analysis was recorded at specific time-points using a custom written software in the ASYST programming environment and saved on the hard disk of a Dell 425E computer.

Hemodynamic calculations

The flows at the PA, PV and HA were indexed by dividing by body weight and expressing them as the cardiac index (CI), portal venous flow index (PVFI) and hepatic artery flow index (HAFI), respectively.

The following variables were calculated:

1. Total hepatic flow index (THFI) (ml/min/kg) = HAFI + PVFI
2. Oxygen content (ml/ml) = Fractional SO_2 x 1.34 (ml/g) x [Hb] g/ml

   (SO_2 is specific for each blood vessel)
3. Oxygen delivery (DO_2) (ml/min/kg) = Flow index (ml/min/kg) x Oxygen content
4. Oxygen consumption ($\dot{V}O_2$) (ml/min/kg) = Flow index (ml/min/kg) x [Arterial oxygen content – Venous oxygen content].
5. Systemic oxygen extraction (EO_2) (%) = ($SaO_2 - SvO_2$) ÷ SaO_2
6. Hepatic oxygen extraction (HEO_2) (%) = [((HA SO_2 x HAFI/THFI) + (PV SO_2 x PVFI/THFI)) – HV SO_2] ÷ [(HA SO_2 x HAFI/THFI) + (PV SO_2 x PVFI/THFI)]

The percentage of the changes in oxygen delivery due to each of hemoglobin oxygen saturation, flow and hemoglobin levels were calculated by comparing the values at baseline and at the end of the experiment in equations 2 and 3 above.

In calculating the oxygen content, dissolved oxygen was ignored as it is a minimal component of oxygen delivery. The dissolved oxygen content (ml O_2 /ml blood) is 3.1 x 10^{-5} x pO_2 (mmHg).

Blood and tissue samples

The total volume of blood samples was limited to 15 ml (~8% of circulating volume), to avoid significant hypovolemia or anemia. Sample aliquots were replaced with an equal volume of Ringer's lactate. Heparinized samples were taken from pre-hepatic (aorta and PV), post hepatic (hepatic vein) and central venous catheters for measurement of the systemic and hepatic oxygen metabolism. Samples were taken at multiple time points including 0 min (baseline), 60 min, 120 min, 180 and 240 min. Blood gas measurements (ABL 500 blood gas analyzer, Radiometer, Copenhagen) and co-oximetry (Model OSM 3 Co-oximeter, Radiometer) were performed immediately after sampling. Arterial or venous plasma for lactate levels was immediately processed and saved at –80°C until subsequent assay. Immediately after euthanization, a wedge biopsy was taken from the liver and snap frozen in liquid nitrogen for later assays.

To quantify the lactate, the plasma was centrifuged (10 min 10,000g), and the supernatant was subjected to enzyme linked metabolite assay with measurement of NADH levels by spectrophotometry at 340 nm.[23] Net lactate balance across the liver was calculated:

Lactate balance (μmol/min/kg) = Lactate delivery – Lactate output

= [(Arterial lactate mmol/l x HAFl l/min/kg) + (PV lactate mmol/l x PVFl l/min/kg)] – (HV lactate mmol/l x THFl l/min/kg)

Liver alanine and aspartate transaminases were assessed by standard medical laboratory automated techniques.

Histology samples were embedded in paraffin. Liver samples were stained with hematoxylin and eosin, and examined by a pathologist blind to the group of the piglet. Liver damage was scored semi-quantitatively (Table 2-1) using the degree of necrosis, sinusoid congestion and neutrophil infiltration.[24] For necrosis, congestion and neutrophil infiltration a score of 0-4 was assigned to the zone examined (peri-portal, intermediate, peri-venular). Necrosis was defined by loss of nuclear detail and loss of well defined cell borders. Congestion was defined as the engorgement of venules or sinusoids with red cells, platelets or fibrin. Neutrophil infiltration was scored according to percentage of infiltration in peri-venular and peri-portal zones, and by the number of neutrophils per high power field in the intermediate zone.

Statistical analysis

Results are expressed as mean ± standard error of the mean. Data were analyzed with one-way ANOVA testing for differences from baseline. Non-parametric equivalents were used if tests for both normality and equal variance failed. Fisher LSD or Dunn's test was used for post-hoc analysis. The threshold for significance was $\alpha < 0.05$. A statistics package (Sigma Stat 2.0, Jandel Scientific, San Rafael, CA) was used.

Score	Extent of tissue necrosis or congestion	Degree of perivenular and periportal zone infiltration by neutrophils	Neutrophil infiltration into intermediate zone (Neutrophils/hpf)
0	<1%	<10%	<1
1	1% - 10%	Mild infiltrate in 11 – 50%	1 – 10
2	11% - 25%	Mild infiltrate in >50%	11 – 20
3	26% - 50%	Dense infiltrate in >50%	21 - 50
4	>50%	Confluent infiltrate in>50%	>50

Table 2-1: Scoring of necrosis, vascular congestion and neutrophil infiltration of post H-R liver.[24]

Results

Hemodynamic profile (Table 2-2)

There were no significant changes in the systemic parameters of CI, heart rate or MAP, during the 4 h of observation. The flow indexes for the PV, HA and the THFI also showed no significant changes during the experiment. Boluses of 96 ± 17 ml/kg (24 ml/kg/h) of Ringer's lactate were used as described, to maintain the CVP and CI within the defined parameters.

Hemoglobin levels and saturation

Mean hemoglobin at 240 min (8.5 ± 0.8 g/dl) was lower but not significantly different from baseline (10.5 ± 0.8 g/dl). Hemoglobin oxygen saturation was not significantly changed in any vessel although those of the HV and PV were lower at the end of the experiment (Table 2-3).

Oxygen metabolism (Table 2-3)

The PV DO_2 deteriorated at 120 min of the experiment ($p<0.05$). It remained significantly less than baseline for the rest of the observation period. An estimated 43% of this fall was due to a reduced PV SO_2, 37% to the observed reduction in flow and 20% to the reduced hemoglobin level. The HA DO_2 did not change significantly from baseline. The hepatic oxygen extraction and consumption did not show significant changes.

Blood gas and lactate analysis (Table 2-3)

Arterial pH and arterial plasma lactate did not significantly change during the observation period. The p_aCO_2 remained stable, although some variability in p_aO_2 was noted which did not significantly affect oxygen delivery. After 240 min of observation there was also no significant difference between the arterial lactate (2.3 ± 0.2 mmol/l), PV lactate (1.9 ± 0.3 mmol/l) or hepatic venous lactate (1.9 ± 0.3 mmol/l) concentrations. The net lactate balance across the liver was 4 ± 8 µmol/min/kg.

Measurement of plasma transaminases

The alanine transaminase at baseline and at the end of the experiment were 36 ± 2 IU/l and 26 ± 5 IU/l respectively. The values for the aspartate transaminase were 75 ± 18 IU/l and 47 ± 11 IU/l respectively. No significant differences were noted.

Liver histology

The semiquantitative assessment of histologic damage showed no cell necrosis or sinusoid congestion. The scores for neutrophil infiltration out of a maximum of 4 were 0.5 ± 0.3 in peri-portal, 1 ± 0.4 in the peri-venular and 0.75 ± 0.5 in the intermediate zones.

	0 min	60 min	120 min	180 min	240 min
Cardiac Index	128 ± 9	103 ± 16	102 ± 21	106 ± 16	117 ± 18
Heart Rate	223 ± 15	223 ± 19	220 ± 14	246 ± 21	261 ± 12
MAP	78 ± 9	66 ± 6	59 ± 6	72 ± 10	57 ± 10
HAFI	6 ± 2	5 ± 2	6 ± 2	8 ± 2	11 ± 3
PVFI	38 ± 3	31 ± 4	27 ± 4	31 ± 4	24 ± 4
THFI	41 ± 3	33 ± 4	30 ± 4	35 ± 3	32 ± 2

Table 2-2: Systemic and hepatic hemodynamics during 240 min of experimentation. Cardiac index, hepatic artery flow index (HAFI), portal venous flow index (PVFI) and total hepatic flow index (THFI) as ml/min/kg, heart rate as beats/min, mean arterial blood pressure (MAP) as mmHg. Values are Mean ± SEM.

	0 min	60 min	120 min	180 min	240 min
Arterial pH	7.38 ± 0.04	7.37 ± 0.04	7.37 ± 0.02	7.35 ± 0.04	7.36 ± 0.04
p_aO_2	72 ± 4	95* ± 10	88 ± 8	62 ± 7	62 ± 9
p_aCO_2	41 ± 3	39 ± 3	34 ± 2	45 ± 7	51 ± 14
Arterial plasma lactate	1.7 ±0.1	n/a	1.7 ±0.2	n/a	2.3 ±0.2
Arterial SO_2	98 ± 1	96 ± 4	98 ± 1	98 ± 2	95 ± 4
PV SO_2	73 ± 3	75 ± 3	70 ± 8	63 ± 7	54 ± 9
HV SO_2	55 ± 4	48 ± 8	51 ± 8	48 ± 8	42 ± 6
HA DO_2	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
PV DO_2	4.1 ± 0.7	3.1 ± 0.5	2.5* ± 0.4	2.2* ± 0.4	1.3* ± 0.3
Hepatic EO_2	25 ± 5	37 ± 10	32 ± 6	30 ± 8	38 ± 8
Hepatic VO_2	0.8 ± 0.1	1.1 ± 0.3	0.7 ± 0.2	0.9 ± 0.2	0.7 ± 0.3

Table 2-3: Plasma pH, blood gas and hepatic oxygen metabolism changes during 240 min of experimentation.

p_aO_2/p_aCO_2 (mmHg); lactate (mmol/l); oxygen saturation (SO_2) (%); hepatic oxygen consumption (VO_2) and delivery (DO_2) (ml/min/kg); oxygen extraction (EO_2) (%). Hepatic artery (HA), portal vein (PV), hepatic vein (HV). Values are mean ± SEM. * $p< 0.05$ vs baseline.

Discussion

In selecting the piglet and the procedures for this model, consideration was taken of the words of Albert Schweitzer on animal experimenters: "It is the duty of the researcher to ponder in every case whether it is really and truly necessary thus to sacrifice an animal for humanity. They ought to be filled with anxious care to alleviate, as much as possible the pain they cause."

The choice of piglet as a model

Piglet responses to H-R are similar to human infants, and study findings have been applicable to clinical disease. Central nervous system hemodynamic control,[25] and the pulmonary vascular response to hypoxia,[26] are similar to human infants. Infant studies on the myocardial response to perinatal asphyxia[27] and hypothermia as therapy in perinatal asphyxia,[28] confirm the relevance of earlier findings in piglet models.[29] Previous newborn piglet H-R studies have also reported on hepatic oxygen metabolism and hemodynamic changes,[10,22] providing baseline information for further model development. They have shown that the liver is resistant to hypoxia to some degree due to the ability to increase oxygen extraction. The interaction of the liver with other organs in an intact model and the response during reoxygenation had not yet been described in such models.

Previous models

A model of perinatal asphyxia needs to take into account the organ-organ interactions due to the global nature of perinatal asphyxia. Many hepatic studies have

been ischemia-reperfusion models using isolated liver perfusion or clamping the PV in intact animals.[30,31] Piglet models using hypoxia in tandem with carotid occlusion exclude the effect of circulating factors during hypoxia.[32] Such models are not representative of the global ischemia of perinatal asphyxia, a problem which is addressed by our model. Global H-R in newborn rats[33,34] with euthanization at various times during the H-R process can track organ response but there is no continuous monitoring of hemodynamic variables. Larger animal models allow catheterization for hemodynamic monitoring and collection of blood samples from specific vessels. The exteriorized fetal lamb has been used in this respect[35] to study hypoxanthine metabolism during hypoxia and reoxygenation. Bristow et al used exteriorized catheters in a fetus in utero, allowing assessment of hepatic blood flow and glucose metabolism.[36] Oxygen metabolism measurement and hepatic hemodynamics during hypoxia in 9 day old lambs have also been measured using chronic catheterization.[7,8] Such experiments are very close to the clinical scenario. Radionuclide labelled microspheres were used for measuring hemodynamic flow variables, but this technique does not allow continuous monitoring, which is important for a detailed examination of the temporal profile during H-R. We have previously made continuous measurements of hepatic hemodynamics and oxygen delivery, using surgically placed flow probes and intravascular catheters in a newborn piglet model.[10] The present model allows additional measures of hepatic oxygen extraction and consumption. The linking of hemodynamic and oxygenation changes to biochemical changes, and subsequent inflammatory changes, is not well described. The

use of pre- and post-hepatic sampling in the current model allows examination of such changes.

Methods

Initial inhalational anesthesia was simple to administer to a piglet by mask. Isoflurane causes less depression of cardiac output and arterial blood pressure, with no effects on liver metabolism compared to halothane.[37] Inhalational anesthetics were discontinued soon after the establishment of vascular access and mechanical ventilation because isoflurane can be protective against liver injury in H-R.[38] The subsequent combination of opioid (fentanyl) for pain suppression and benzodiazepine (midazolam) for sedation, have been shown to be effective with minimal cardiovascular effects as compared to anesthetic agents. Acepromazine for tranquilization and pancuronium for paralysis are also established safe medications in swine.[39]

Ultrasonic flow probes were used as they provide continuous measurement and also allow quantification of both the arterial and PV hepatic blood supply separately, unlike the technique of using radioactive microspheres.[8] We have previously established the suitability of using the flow probes in similar piglet models,[10,40] and their use has also been extensively verified in a variety of swine models.[41]

The technique for the HV catheter placement has not previously been described. Bristow et al placed an HV catheter in a lamb fetus via a thoracotomy and insertion through the inferior vena cava and verified the positioning by comparing blood oxygen saturations.[36] Our fenestrated 20G catheter was placed under direct vision, and the

position was verified post mortem to ensure it had not been displaced. We were thus able to fully assess hepatic oxygen metabolism.

Hemodynamics and oxygen metabolism

The systemic and hepatic hemodynamics were not significantly altered. The PVFI seems to show a reduction over the time course of the observations but without approaching significance ($p = 0.21$, $\beta = 0.2$). We could speculate that prolonging the observation period would cause a significant reduction.

The only significant change in oxygen metabolism was a reduction in PV DO_2. Interestingly 57% of the fall in PV DO_2 was accounted for by the observed reduction in PVFI and hemoglobin concentration. The PVFI reduction may have been contributed to by the ongoing requirement for fluid infusions above basal requirements. Despite closing the surgical incisions as much as possible and covering with wet saline soaks the fluid loss from the open laparotomy is likely to have been significant. The remaining 43% of the $PVDO_2$ reduction was due to the reduced PV hemoglobin saturation. Since the arterial saturations did not change, this reduction in PV SO_2 implies an increase in oxygen consumption by the intestine. Despite the reduction in PV DO_2 there were no significant changes in hepatic VO_2 or EO_2. The stability of the VO_2 would require an increase in EO_2 or HA DO_2 to compensate for the reduced PV DO_2. We were not able to demonstrate this due to the small sample size in this pilot study.

The $PVDO_2$ and PVFI deterioration may have been avoided with a longer pre-experimental stabilization period as the measurements observed were more stable after 60

min of the experiment. A chronically instrumented model with full recovery from surgery prior to hypoxia would be an ideal alternative.

Lactate and transaminases

Neither of these parameters demonstrated changes over the course of the experiment. This confirmed the adequacy of oxygenation of the model, the lack of hepatocellular injury and the feasibility of assessing the hepatic lactate balance.

Histology

The lack of histologic change is expected as neutrophil infiltration can take up 6-12 hours to be evident after an insult[42]. It confirms that surgery has not markedly confounded results, as from the commencement of surgery to the end of reoxygenation was usually ~6 hours. Intermittent liver biopsies would be possible with the model to monitor tissue histologic and biochemical changes.

Limitations

Despite approximating the incisions using wet saline soaks there was an ongoing requirement for fluid infusions above the basal requirements of 15 ml/kg/h. This is a known complication of keeping a laparotomy open for prolonged periods. It was probably a contributing factor to the mild metabolic acidosis in the initial piglets. The placement of the hepatic artery flow probe was also technically difficult due to the small size of the vessel, and the common occurrence of anomalous left or right hepatic arteries. Umbilical venous catheter positioning has also been a concern in some studies with

hepatic damage noted.[43] Our histology confirms this was not a problem in our model probably due to the placement of the UVC at the junction of the portal vein and umbilical vein by direct vision.

Conclusions

We have developed a neonatal model, which remained stable through a surgical period and 4 hours of observation in all parameters except portal venous oxygen delivery. It proved suitable for continuous monitoring of hemodynamics, intermittent assessment of oxygen metabolism, pre- and post-hepatic comparisons of plasma markers and post euthanasia tissue assays. The model will thus allow assessment of the effect of whole animal hypoxia on these hepatic parameters.

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Chapter 3

Resuscitation with 21% and 100% oxygen after asphyxia: the effect on perfusion and oxygen metabolism in the neonatal liver

Hepatic hemodynamics in H-R

Introduction

Neonatal asphyxia is associated with hypoxia-reoxygenation (H-R) injury of various degrees in different organs,[1,2] and both hypoxia and reoxygenation contribute to the injury.[3-5] The effects of H-R in different organs have been studied in various animal models. Systemic vasodilatation can occur in tandem with pulmonary hypertension,[6] increased blood flow and extraction of oxygen can maintain oxygen consumption in the brain more so than in the intestine.[7] Although the liver is relatively resistant to H-R,[8] cellular injury may cause hepatic dysfunction,[9] or affect the function of other organs.[10,11] The hepatic sinusoidal endothelial cell is particularly prone to reoxygenation injury,[12] affecting blood flow and organ oxygenation. Liver cell necrosis is associated with oxidative stress.[13] Isolated liver ischemia reperfusion injury has been well described as it is relevant to hepatic surgery,[14] but less is known of hepatic H-R in intact animals. In a model of hypoxia in fetal lambs, Bristow et al showed reduced hepatic blood flow, and oxygen delivery and consumption during hypoxia.[15] The temporal and differential hemodynamic changes during both hypoxia and reoxygenation are less completely described.

The exacerbation of hypoxic injury during reoxygenation is, in part, due to oxidative stress induced injury.[16,17] The concentration of oxygen used in resuscitation may also contribute to H-R injury. There is higher acute[18] and short term[19] oxidative stress if 100% rather than 21% oxygen is used in asphyxiated infants. In addition 21% oxygen seems as effective as 100% in terms of acute clinical responses.[20,21] The clinical

follow up of infants also demonstrates similar neurodevelopmental outcomes at 18-24 months.[22]

To our knowledge the hepatic responses to reoxygenation with either 21% or 100% oxygen have not previously been described in the asphyxiated newborn. Therefore, we designed an experiment to continuously record the temporal responses of liver hemodynamics and oxygen and lactate metabolism, during severe hypoxia, and then reoxygenation with 100% or 21% oxygen, in an intact newborn animal model. We hypothesized that the recovery of liver perfusion and oxygen metabolism after severe hypoxia would be as effective using 21% as 100% oxygen.

Methods

The study conformed to the regulations of the Canadian Council of Animal Care (Revised 1993) and was approved by the Health Sciences Animal Welfare Committee, University of Alberta.

Animals

The animals were newborn mixed breed piglets between 1-3 days old and 1.5-2.0 kg in weight. They were obtained from local breeders on the day of experimentation. Their weights were determined and they were observed for 30 min to allow for accommodation. Twenty-seven animals were block randomized. Experimental animals underwent hypoxia and reoxygenation with either 21% (n=9) or 100% (n=9) oxygen. Controls (n=9) underwent surgery, but not hypoxia or reoxygenation.

Animal preparation

The animals were placed on a radiant warmer (Ohio NC neonatal care center). Anesthesia was induced with isoflurane (5%) and oxygen mixture at 3 l/min via a sealed mask for 3 min. Halothane was not used due to the possibility of hepatotoxicity. Anesthetic gases were delivered by a VMS Fraser-Harlater anesthesia machine. The Isoflurane was reduced to 2% for maintenance anesthesia. The animals continued to breathe spontaneously. With the establishment of venous access, inhalational anesthesia was discontinued and followed by a bolus of fentanyl (10 μg/kg) and of acepromazine

(0.1 mg/kg) intavenously. Intravenous (IV) infusions of fentanyl at 5-10 μg/kg/h, midazolam at 0.1-0.2 mg/kg/h, and pancuronium at 0.05-0.1 mg/kg/h were commenced. Further pancuronium (100 μg/kg) or fentanyl (10 μg/kg) boluses were given as needed, for muscle paralysis and analgesia respectively.

Via a groin incision 5 Fr single lumen catheters (Sherwood Medical Co., St Louis, MO) were inserted in the femoral artery and vein and sutured in place. The arterial line was used to monitor blood pressure and for blood sampling. The venous line was used for IV anesthesia and maintenance infusion fluids. A 5 Fr single lumen (Sherwood Medical Co.) right external jugular catheter was positioned for central venous blood sampling and pressure (CVP) measurement.

A 3.0 mm endotracheal tube (Mallinckrodt, St Louis, MO) was inserted into a tracheostomy. With the onset of paralysis assisted ventilation with a neonatal ventilator (Model IV-100, Sechrist Inc., Anaheim, CA) was commenced. The inspired oxygen concentration (FiO_2) was monitored with an in-circuit oxygen analyzer (Ohmeda S100, Ohmeda Medical, Laurel, MD). Baseline ventilation was pressure controlled at 16/4 cm H_2O, at 12-18 breaths per min with an FiO_2 of 0.21-0.25.

Via a left anterior thoracotomy, a 6 mm ultrasonic flow probe (6SB Transonic, Ithaca, NY) was placed around the main pulmonary artery (PA). A laparotomy was then performed. The porta hepatis was dissected, 1 mm and 3 mm ultrasonic flow probes (1SB and 3SB, Transonic, respectively) were placed around the proper hepatic artery (HA) and portal vein (PV), respectively. All flow probes were zero calibrated prior to use. The

umbilical vein was catheterized intraperitoneally with a 5 Fr single lumen catheter (Sherwood Medical Co.), with the tip placed at the junction with the left PV under direct vision. The liver was then mobilized with minimal handling by taking down the falciform ligament. The right hepatic vein was identified and an 18 G IV cannula (B-D, Franklin Lakes, NJ) was inserted and secured.

The laparotomy and thoracotomy wounds were loosely closed and covered with warm saline soaks. The duration of the surgical procedure was 60-70 min.

A dextrose-saline infusion at 15 ml/kg/h maintained blood glucose levels and hydration. Sodium bicarbonate was infused at 0.25 mmol/kg/h in all animals. This corrected a mild metabolic acidosis that resulted from the procedure. To avoid hypovolemia an IV bolus of Ringer's lactate (10 ml/kg) was given at the time of initial paralysis, at jugular line insertion and prior to the laparotomy. Further boluses of 10 ml/kg were given outside of the stabilization period, if the CVP fell below 5 cm H_2O or if PA flow was <120 ml/min.

Stabilization protocol

The period of post-operative stabilization was 60 min. An initial arterial blood gas was performed to adjust the ventilation to maintain the p_aO_2 at 60-80 mmHg; the p_aCO_2 at 35-45 mmHg and the pH at 7.35-7.45. Any base deficit of greater than 5 was corrected at this point with 4.2% sodium bicarbonate. The hemodynamic measurements of flow were required to be stable with less than 10% change during the last 15 min of the stabilization period. Rectal temperature was kept at 38.5-39.5°C.

Experimental protocol

Severe hypoxia was induced by reducing the FiO_2 to 0.12, with the addition of nitrogen to the ventilator gases. This was then adjusted, to achieve an arterial pO_2 of 30-40 mmHg. This correlated to transcutaneous hemoglobin saturations of 40-50%. If the PA flow fell below 100 ml/min or to 1/3 the baseline value, whichever was lower, the FiO_2 could be increased to a maximum of 0.14 to allow a 120 min period of hypoxia to be achieved. At the end of the 120 min hypoxic period the FiO_2 was abruptly increased to 100% or 21% according to the randomization of the piglets. This was maintained for 60 min. At this time those in the 100% group had their oxygen reduced to 21%. After a further 60 min in 21% oxygen the piglet was euthanized with an IV injection of pentobarbital (240 mg), and the liver tissue harvested for subsequent analysis. Control piglets were ventilated with 21% oxygen throughout, a total of 240 min.

Hemodynamic measurements and recording

Heart rate, via a 3-lead ECG, mean arterial blood pressure (MAP) and CVP were continuously monitored using a Hewlett Packard cardiomonitor (Model HP 78833B). Transcutaneous arterial oxygen saturation (S_aO_2) was monitored with a pulse oximeter (Nellcor, Hayward, CA). The blood flows at the PA, HA and PV were also continuously monitored throughout the study period. The analogue outputs of these hemodynamic measurements were digitized with a DT2801-A analogue to digital converter (Data translation, Ontario, Canada). Data for analysis was recorded and averaged over a 2

minute period at specific time-points using custom written software in the ASYST programming environment and saved on the hard disk of a Dell 425E computer.

Blood and tissue sampling

The total volume of blood sampling was limited to 15 ml (~8% of circulating volume), to avoid significant hypovolemia or anemia. Heparinized samples were taken from pre-hepatic (aorta and PV), post hepatic (hepatic vein) and central venous catheters for ascertainment of changes in systemic and hepatic oxygen metabolism. Samples were taken at baseline, just prior to hypoxia (time 0 min), at 60 min of hypoxia and at the end of hypoxia prior to reoxygenation (120 min), then at 5, 15, 60 and 120 min of reoxygenation. Control samples were taken at the corresponding times of baseline, 60, 120, 125, 135, 180 and 240 min. Blood gas measurements (ABL 500 blood gas analyzer, Radiometer, Copenhagen) and co-oximetry (Model OSM 3 Co-oximeter, Radiometer) were performed immediately after sampling. Arterial plasma was immediately processed and saved at –80°C until subsequent assay for lactate levels. Immediately after euthanization, a wedge biopsy was taken from the liver and snap frozen in liquid nitrogen for lactate measurement.

Analysis of lactate

Frozen tissue was extracted with 6% perchloric acid and centrifuged. The neutralized supernatant was subjected to enzyme linked metabolite assay with measurement of NADH levels by spectrophotometry at 340 nm to quantify the lactate.[23]

Tissue protein levels were measured using a bovine serum albumin standard to standardize the lactate values obtained. [24] Plasma lactate was assayed similarly, without extraction.

Hemodynamic calculations

The flows at the PA, PV and HA were divided by body weight and expressed in ml/min/kg, as the cardiac index (CI), portal venous flow index (PVFI) and hepatic artery flow index (HAFI), respectively.

The following variables were calculated:

1. Total hepatic flow index (THFI) (ml/min/kg) = HAFI + PVFI
2. Oxygen content (ml/ml) = Fractional SO_2 x 1.34 (ml/g) x Hb (g/ml)
   (SO_2 is specific for each blood vessel)
3. Oxygen delivery (DO_2) (ml/min/kg) = Flow index (ml/min/kg) x Oxygen content
   (Flow Index and oxygen content are specific for each blood vessel)
4. Oxygen consumption ($\dot{V}O_2$) (ml/min/kg) = Flow index (ml/min/kg) x Arterial oxygen content – Venous oxygen content].
5. Systemic oxygen extraction (EO_2) (%) = $(S_aO_2 - S_vO_2) \div S_aO_2$
6. Hepatic EO_2 (%) = [((HA SO_2 x HAFI/THFI) + (PV SO_2 x PVFI/THFI)) – HV SO_2] ÷ [(HA SO_2 x HAFI/THFI) + (PV SO_2 x PVFI/THFI)]

Statistical analysis

Results are expressed as mean ± standard error of the mean. Data were analyzed by two-way ANOVA, then one-way ANOVA testing for differences within or between groups. Non-parametric equivalents were used if tests for both normality and equal variance failed. Fisher LSD or Dunn's test was used for post-hoc analysis. The threshold for significance was $\alpha < 0.05$. A statistics package (Sigma Stat 2.0, Jandel Scientific, San Rafael, CA) was used.

Results

Systemic responses

At baseline there were no significant differences in the hemodynamic measurements between groups. The baseline p_aO_2 did not differ between groups: 78 ± 6, 75 ± 6 and 76 ± 5 mmHg for control, 21% and 100% groups, respectively. Control piglets were maintained at a mean p_aO_2 of 79 ± 3 mmHg through the 4 h of the study. During hypoxia, the mean p_aO_2 measured at 60 and 120 min were 39 ± 3 mmHg and 37 ± 3 mmHg in the 21% and 100% groups respectively, and the mean S_aO_2 were 47 ± 3 and 41 ± 4% respectively. The H-R groups did not significantly differ. The mean p_aCO_2 throughout the study period did not differ between groups (38 ± 1; 36 ± 1 and 38 ± 2 mmHg in the control, 21% and 100% groups, respectively). Arterial pH (Table 3-1) did not change significantly in the controls. From the end of hypoxia up to 60 min of reoxygenation, the piglets in both H-R groups were acidotic (vs. controls and baseline values), with the lowest pH at 5 min of reoxygenation (6.99 ± 0.03 and 7.00 ± 0.05 in the 21% and 100% group, respectively). The 100% group recovered to baseline at 120 min of reoxygenation but not the 21% group. Intravenous volume support of Ringer's lactate was not significantly different in each group during the 4 h of the study. 116 ± 15; 109 ± 11 and 95 ± 12 ml/kg respectively were given to the control, 21% and 100% groups respectively.

Cardiac index (fig 3-1), MAP (fig 3-2) and heart rate (fig 3-3) did not change significantly, compared to baseline, in the control group over the 240 min of the study.

After 120 min of hypoxia, CI and MAP were significantly decreased in both H-R groups compared to controls and baseline. The CI and MAP in both groups improved dramatically and did not significantly differ from controls or baseline within 5 minutes of reoxygenation. The CI of the 100% group, after 60 min of reoxygenation, and of both H-R groups at 120 min were significantly below baseline. The MAP remained significantly below baseline during reoxygenation in both H-R groups. The heart rate showed a trend to tachycardia at 60 min of hypoxia ($p = 0.07$) compared to baseline, and increased significantly above baseline in the 21% group at, and after, 60 min of reoxygenation.

The systemic DO_2 (fig 3-4) in the controls was significantly less than baseline from 120 min while the systemic EO_2 (fig 3-5) significantly increased from 135 min. There was no change in systemic $\dot{V}O_2$ (fig 3-6).

In piglets undergoing H-R, systemic DO_2 decreased, EO_2 increased and $\dot{V}O_2$ decreased during hypoxia (vs. controls and baseline, $p<0.05$). Upon reoxygenation, systemic DO_2 in 21% and 100% groups increased and did not differ from controls at 5 min and thereafter. The 100% group did not differ from baseline at 5 min of reoxygenation but was reduced thereafter. The 21% group remained significantly below baseline throughout reoxygenation. Systemic EO_2 in the 100% group was significantly below controls and 21% groups until after the change to 21% oxygenation at 60 min of reoxygenation. In the 21% group, systemic EO_2 was significantly above baseline, but not different from controls, throughout reoxygenation. Systemic $\dot{V}O_2$ in the 100% group was significantly below the 21% group at 5 min reoxygenation, then did not significantly

differ between groups. Systemic $\dot{V}O_2$ was significantly below baseline for the first 60 min in the 100% group, and after 120 min of reoxygenation in the 21% group.

Hepatic hemodynamic responses

The 21% and 100% groups did not differ from each other in any hepatic flow index during H-R.

During hypoxia the PVFI (fig 3-7) decreased significantly in the H-R groups compared to controls, and there was a decrease in all groups compared to baseline levels. The HAFI (fig 3-8) did not significantly change in hypoxia. The THFI (fig 3-9) in the H-R groups showed a decreasing trend compared to controls at the end of hypoxia ($p=0.09$, $\beta = 0.3$).

During reoxygenation the THFI and PVFI recovered and were not significantly different from controls or baseline at 5 min in the 21% and 100% groups. The THFI did not significantly differ from baseline in any group, or from controls in the H-R groups thereafter. The PVFI was significantly below baseline in the 21% group and the 100% group from 15 min and at 120 min of reoxygenation respectively, and in the controls after 120 min into the experiment. The HAFI did not differ from baseline or controls in any group.

Hepatic oxygen metabolism

During hypoxia, the total hepatic DO_2 and PV DO_2 (fig 3-10 and 3-11) in the H-R groups were significantly reduced versus controls and baseline, whereas the total hepatic

DO_2 in controls was below baseline from 120 min into the experiment. The HA DO_2 (fig 3-12) was significantly less than controls in the H-R groups at 120 min of hypoxia. The hepatic EO_2 (fig 3-13) in both H-R groups increased significantly during hypoxia compared to baseline and controls. Hepatic $\dot{V}O_2$ (fig 3-14) was significantly reduced in the 100% group compared to controls and baseline by 120 min of hypoxia ($p<0.05$). There was a trend to reduction for the 21% group ($p<0.1$ ANOVA).

During reoxygenation of the 100% group, at 5 min the total hepatic DO_2 and PV DO_2 recovered to baseline. The PV DO_2 was significantly higher than the 21% group and the controls at 5 min (PV DO_2, $p<0.05$), while the total hepatic DO_2 differences approached significance ($p=0.05$ ANOVA). The total hepatic DO_2 in the 100% group then dropped and was significantly below baseline at 120 min. In the 21% group, total hepatic DO_2 and PV DO_2 remained significantly below baseline throughout the reoxygenation phase and in the controls remained below baseline from 120 min into the experiment. There was no difference between groups or from baseline in HA DO_2 by 5 min of reoxygenation. Hepatic EO_2 in the 100% group was significantly lower than the 21% group and baseline at 5 min of reoxygenation. It remained significantly below the 21% group until the switch to 21% oxygen after 60 min but recovered to baseline by 15 min of reoxygenation. In the 21% group, hepatic EO_2 remained significantly above baseline from 15 min, and transiently differed from controls at 60 min of reoxygenation. Upon resuscitation of both H-R groups the hepatic $\dot{V}O_2$ recovered and was not significantly below controls after 5 min of reoxygenation. The hepatic $\dot{V}O_2$ did not significantly differ

from baseline by 5 min of reoxygenation in either H-R group. There was no difference in hepatic $\dot{V}O_2$ between the 21% and 100% groups at any point during H-R.

Plasma and tissue lactate levels

At the end of the experiment, the plasma lactate level of the control piglets was slightly raised and differed significantly versus baseline ($p<0.05$). Both H-R groups had markedly raised plasma lactate levels by the end of hypoxia and throughout reoxygenation. They were significantly higher than controls or baseline, with no difference between 100% and 21% groups (Table 3-1). The liver tissue lactate in the 21% group, but not in the 100% group, was significantly higher than controls after 120 min reoxygenation (fig 3-15).

	0 mins Baseline	120min Hypoxia	15 min Reoxygenation	120 min Reoxygenation
pH				
Control	7.39 ± 0.03	7.37 ± 0.02	7.39 ± 0.02	7.38 ± 0.03
21%	7.40 ± 0.01	7.02 ± 0.04*†	7.05 ± 0.03*†	7.26 ± 0.03†
100%	7.41 ± 0.02	7.00 ± 0.05 *†	7.03 ± 0.04*†	7.30 ± 0.05
Lactate (mmol/l)				
Control	1.8 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	2.4 ± 0.2†
21%	1.9 ± 0.2	8.6 ± 0.6*†	8.0 ± 0.6*†	7.0 ± 0.8*†
100%	2.1 ± 0.3	8.5 ± 0.9*†	7.9 ± 0.9*†	5.4 ± 1.5*†

Table 3-1: Arterial pH and plasma lactate levels during hypoxia and reoxygenation with 21% or 100% oxygen.

Controls did not undergo hypoxia or reoxygenation

($p<0.05$ * versus controls, † versus baseline). Values are mean ± SEM.

Cardiac Index

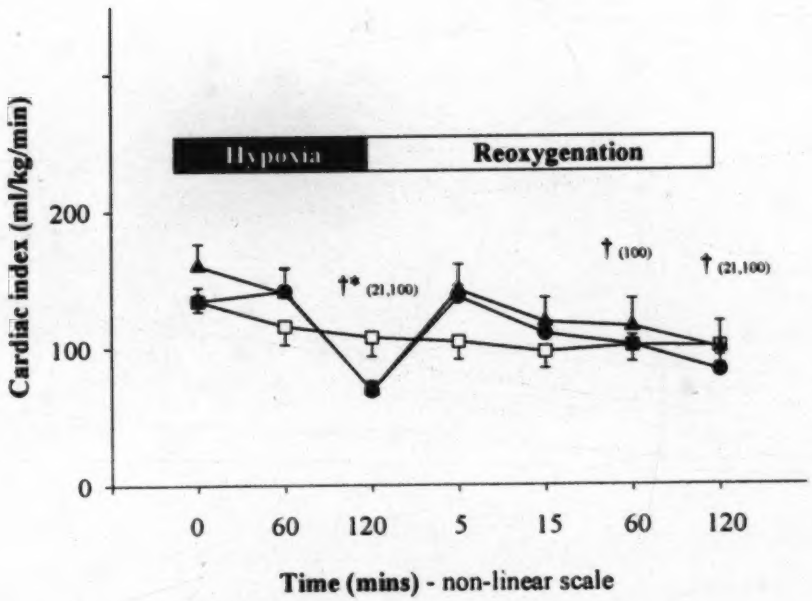

Figure 3-1. Cardiac index during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation.
21=21% group; 100=100% group. $p<0.05$: * vs. controls; † vs. baseline. Values are means ± SEM.

Mean Arterial Pressure

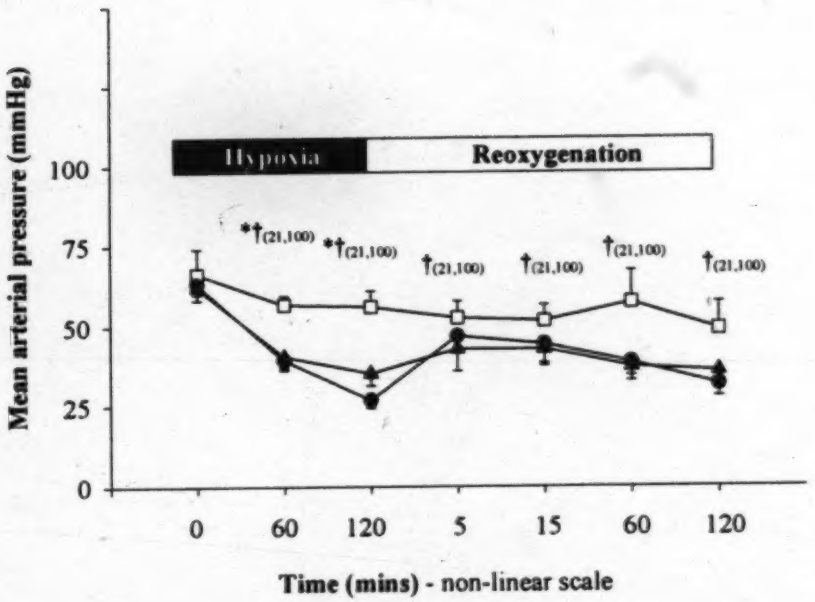

Figure 3-2. Mean arterial pressure during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. 21=21% group; 100 =100% group. $p<0.05$ * vs. controls; † vs. baseline. Values are means ± SEM.

Heart Rate

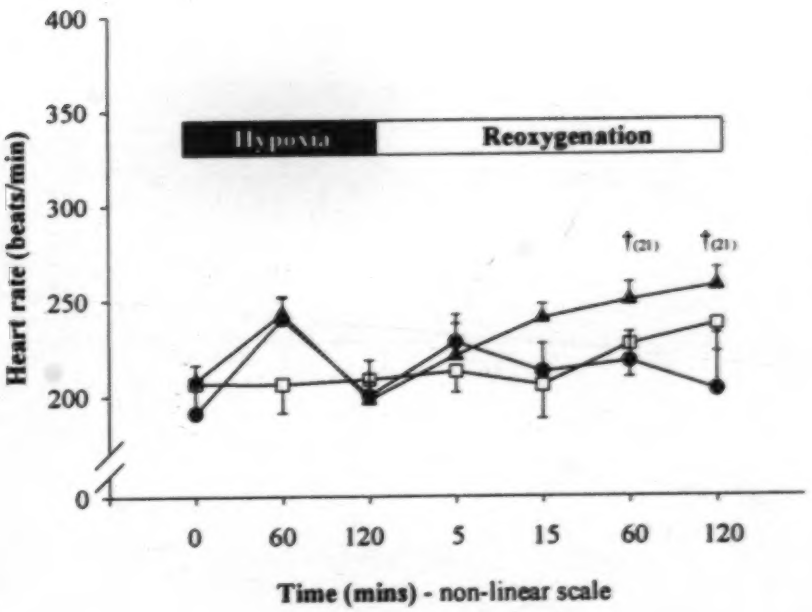

Figure 3-3. Heart Rate during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation.
21=21% group. $p<0.05$ † vs. baseline. Values are means ± SEM.

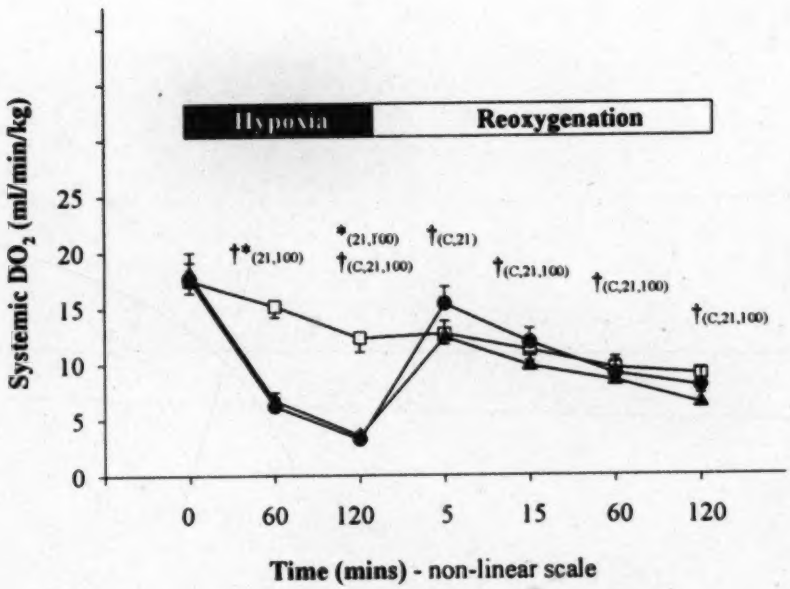

Figure 3-4. Systemic oxygen delivery during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. C = controls; 21=21% group; 100=100% group. $p<0.05$: * vs. controls; † vs. baseline. Values are means ± SEM.

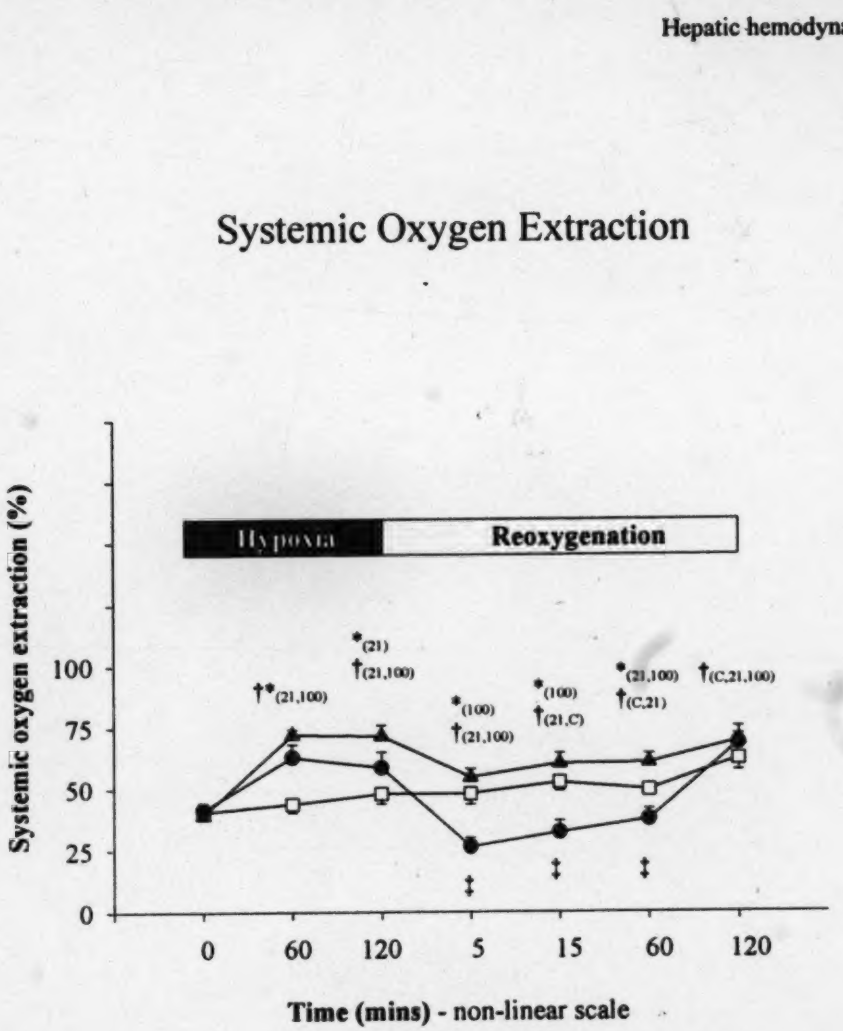

Figure 3-5. Systemic oxygen extraction during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. C = controls; 21=21% group; 100=100% group. $p<0.05$: * vs. controls; † vs. baseline; ‡ 100% vs. 21%. Values are means ± SEM.

Systemic Oxygen Consumption

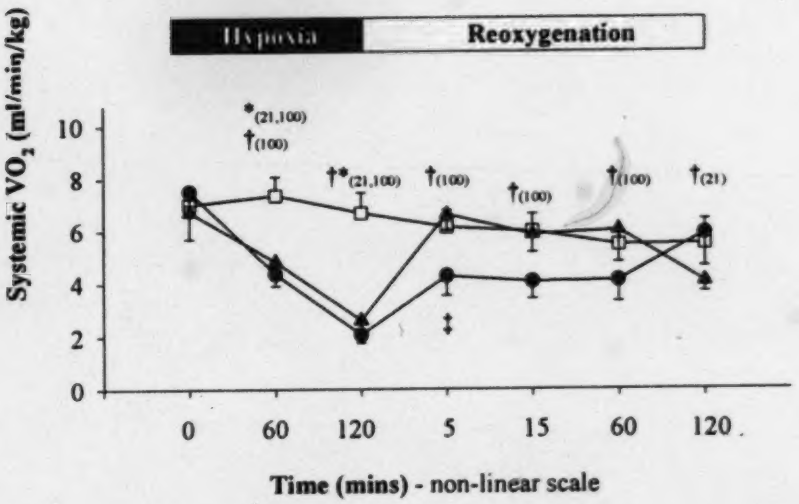

Figure 3-6. Systemic oxygen consumption (VO_2) during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. 21=21% group; 100=100% group. $p<0.05$: * vs. controls; † vs. baseline; ‡ 100% vs. 21%. Values are means ± SEM.

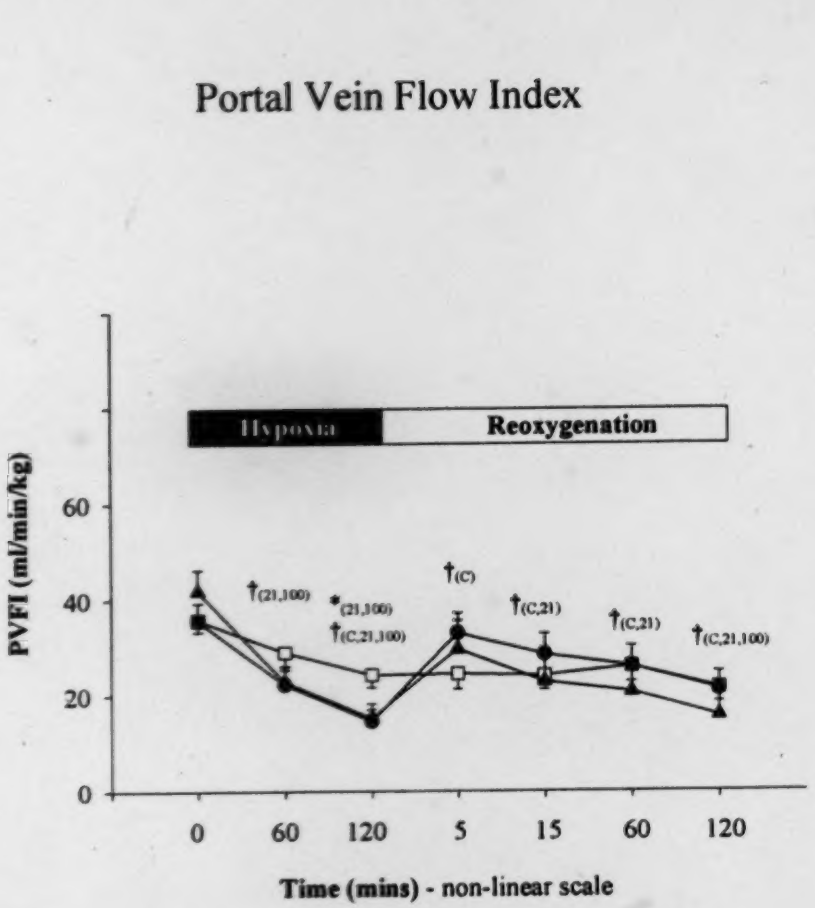

Figure 3-7. Portal vein flow index (PVFI) during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. C = controls; 21=21% group; 100=100% group. $p<0.05$: * vs. controls; † vs. baseline. Values are means ± SEM.

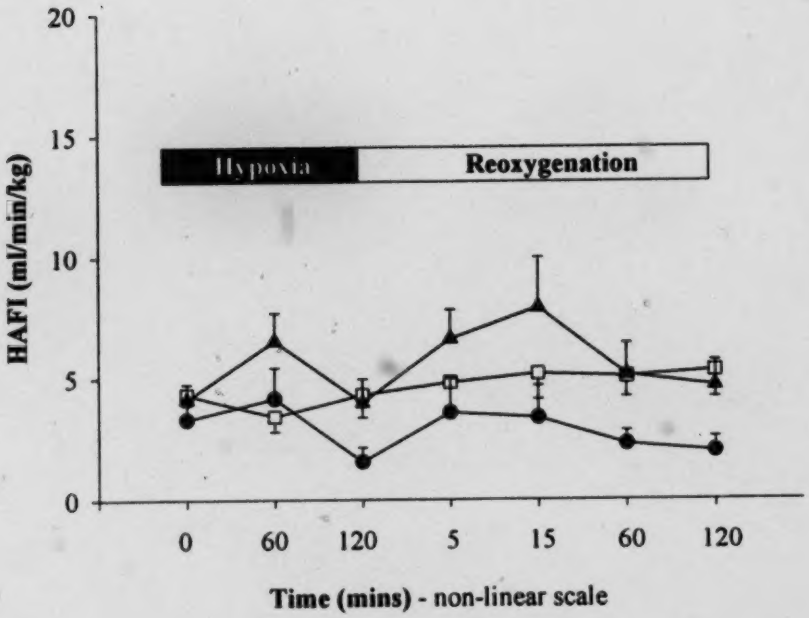

Figure 3-8. Hepatic artery flow index (HAFI) during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. Values are means ± SEM.

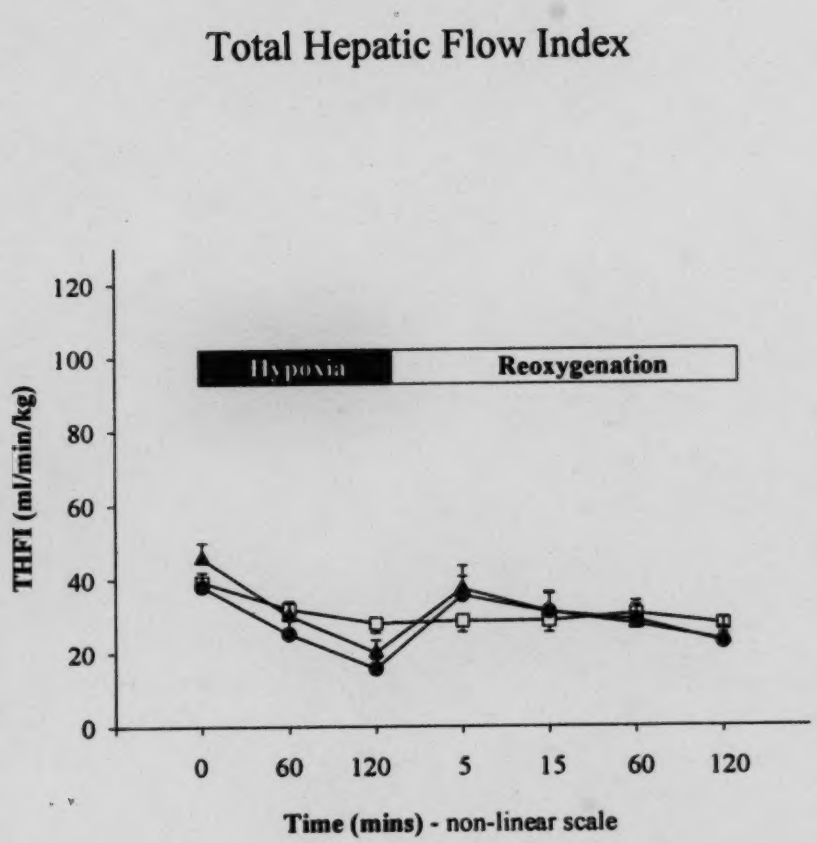

Figure 3-9. Total hepatic flow index (THFI) during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. Values are means ± SEM.

Total Hepatic Oxygen Delivery

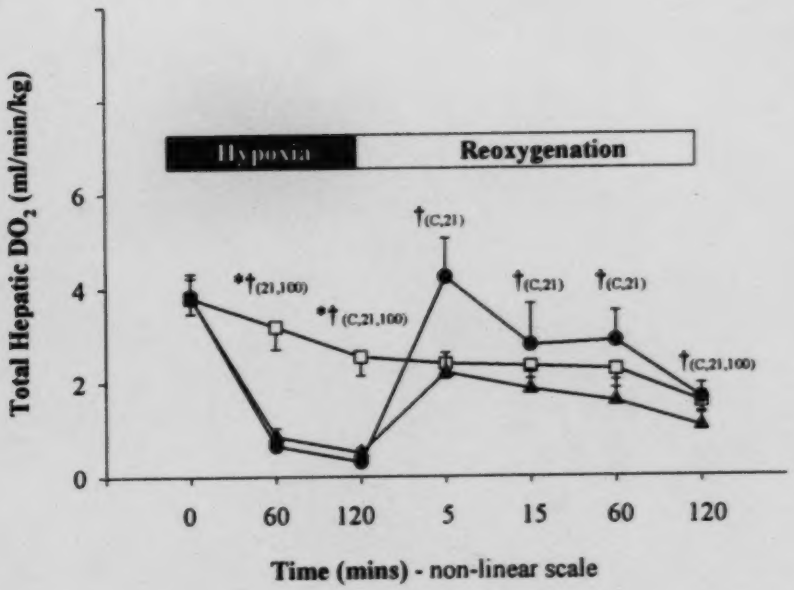

Figure 3-10. Total hepatic oxygen delivery (DO_2) during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. C = controls; 21=21% group; 100=100% group. $p<0.05$: * vs. controls; † vs. baseline. Values are means ± SEM.

Portal Vein Oxygen Delivery

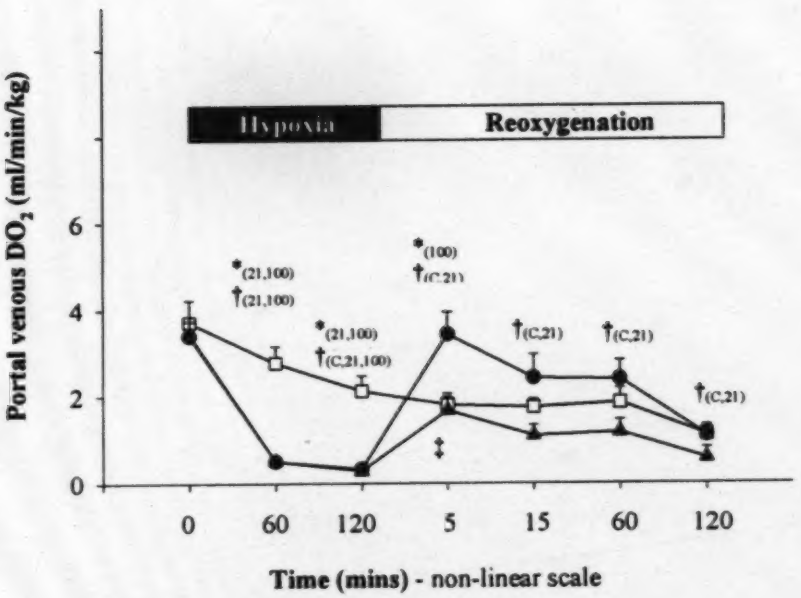

Figure 3-11. Portal vein oxygen delivery (DO_2) during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. C = controls; 21=21% group; 100=100% group. $p<0.05$: * vs. controls; † vs. baseline, ‡ 100% vs. 21%. Values are means ± SEM.

Hepatic Artery Oxygen Delivery

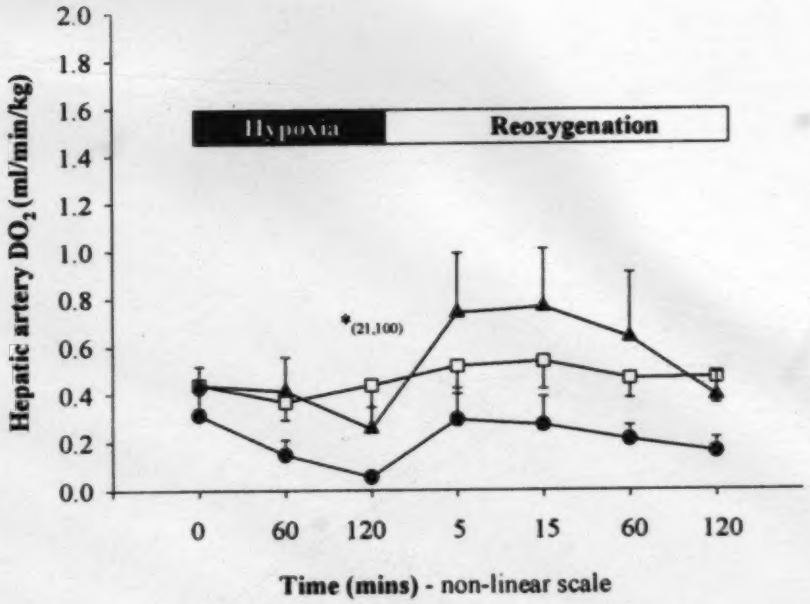

Figure 3-12. Hepatic artery oxygen delivery (DO_2) during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. 21=21%; group, 100=100% group. $p<0.05$: * vs. controls. Values are means ± SEM.

Hepatic Oxygen Extraction

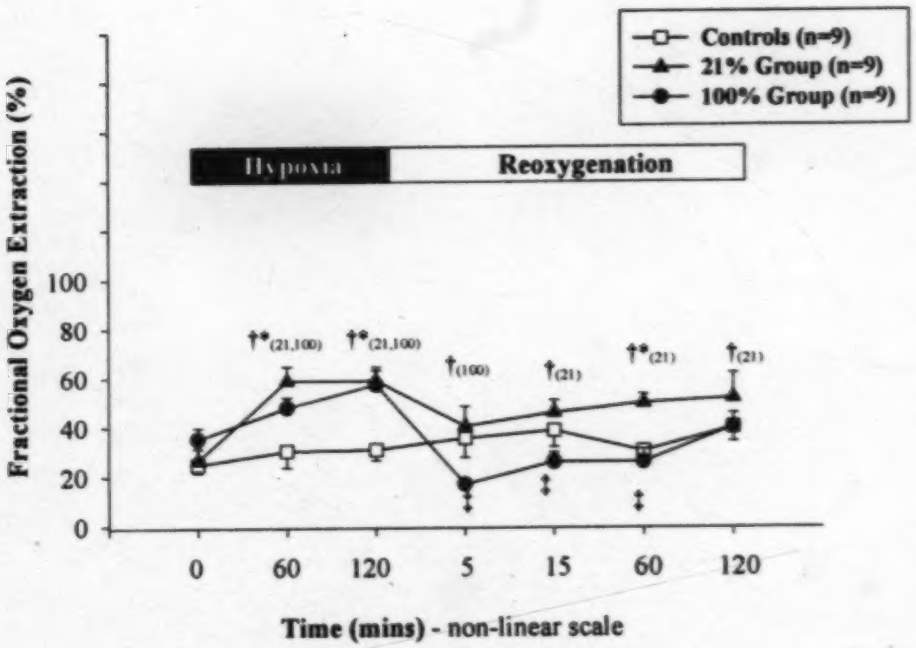

Figure 3-13. Hepatic oxygen extraction during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. 21=21% group; 100=100% group. $p<0.05$: * vs. controls; † vs. baseline; ‡ 100% vs. 21%. Values are means ± SEM.

Hepatic Oxygen Consumption

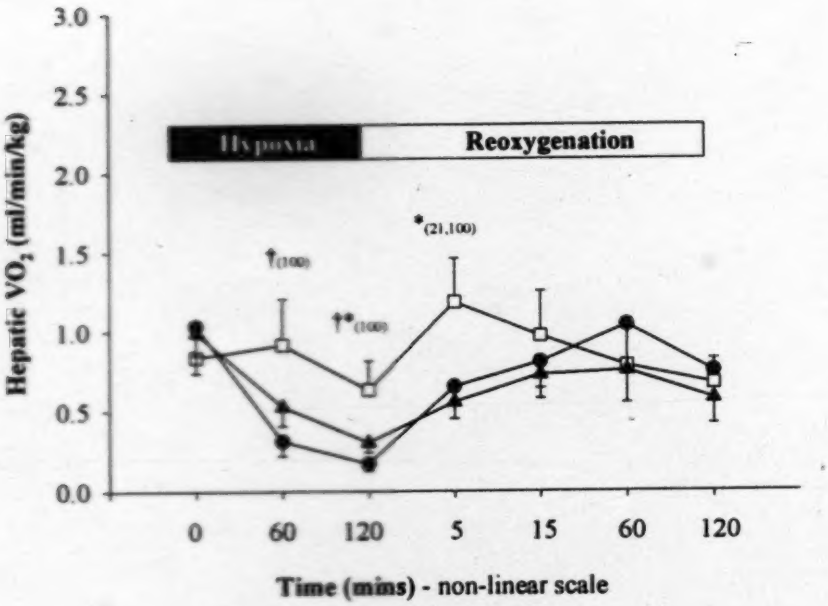

Figure 3-14. Hepatic oxygen consumption (VO_2) during hypoxia and reoxygenation with 21%(▲) or 100%(●) oxygen compared to controls (□) which did not undergo hypoxia or reoxygenation. 21= 21% group;100=100% group. $p<0.05$: * vs. controls; † vs. baseline. Values are means ± SEM.

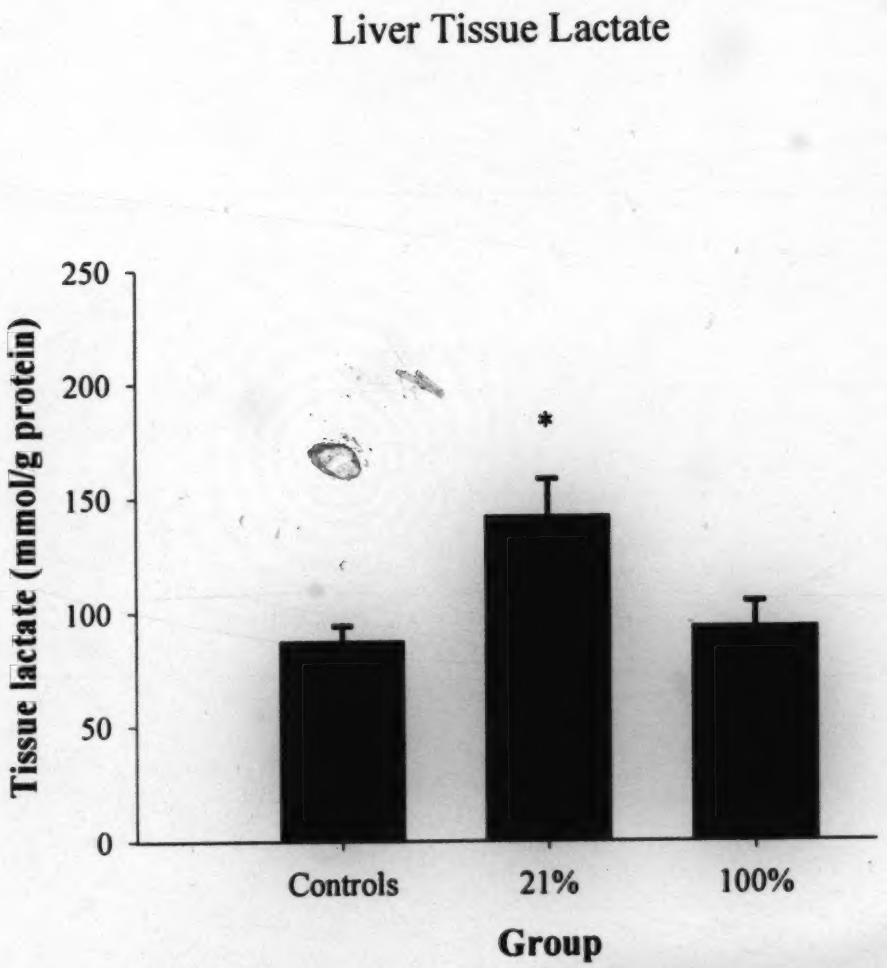

Figure 3-15. Hepatic tissue lactate after hypoxia and 120 min reoxygenation with 21% or 100% oxygen. Controls did not undergo hypoxia or reoxygenation.
$p<0.05$: * vs. controls. Values are means ± SEM.

Discussion

In this study temporal changes in systemic hemodynamics were in line with previous studies during both hypoxia and reoxygenation, and were compatible with a severe hypoxic insult. While the hepatic hemodynamic responses were equivalent for both types of reoxygenation, we showed contrasting hepatic oxygen metabolism with the different oxygen concentrations used in resuscitation: 100% oxygen allowed greater oxygen delivery from the PV than 21% oxygen, but similar oxygen consumption associated with a decreased oxygen extraction. The hepatic lactate levels, however were higher in the 21% group at the end of the experiment.

Hepatic response to hypoxia

The hypoxic piglets showed a significant reduction of the PVFI compared to controls and baseline. This was a greater reduction than in our previous model of hypoxemia,[6] or that reported in neonatal lambs.[25] This is explained by the relatively low pH and the reduction in CI we observed, indicating that the model is behaving similarly to other neonatal models of severe hypoxia which did not report hepatic responses.[26,27] Previous hepatic observations in newborn piglets have shown an increase in the HAFI during moderate hypoxia.[6] The HAFI did not increase in our study but neither did it fall. The THFI showed a similar pattern of change to the PVFI but the effect was attenuated by the lack of change in HAFI. As previously reported, the PVFI is more susceptible to hypoxic flow reduction,[25] but a hepatic artery buffer response can compensate for this to

some degree.[28] Our results suggest reduced effectiveness of this buffer response during severe hypoxia. Flow reductions due to a model effect were also observed which did not, however, seem to mask the effects of H-R.

The total hepatic and PV DO_2 were reduced in both H-R groups, compared to controls and baseline. HA DO_2 was reduced compared to controls. These reductions were due to decreased flow and hemoglobin saturation and were sufficient to cause a fall in hepatic $\dot{V}O_2$, despite increases in hepatic EO_2. Due to compensatory increases in EO_2, the newborn's liver can maintain VO_2 even with 50% reduction in flow.[25] This compensation is insufficient once DO_2 falls below a critical level, which is 2 ml/kg/min in young lambs.[29] Our figures would suggest that newborn piglets have similar requirements for DO_2 (fig 3-11 and 3-12). The hepatic $\dot{V}O_2$ of all the hypoxic piglets was 0.33 ml/kg/min after 120 min of hypoxia from a baseline of 1.2 ml/kg/min, which are also similar to those reported for fetal lambs.[15]

Hence, severe hypoxia is associated with a reduction in hepatic perfusion mostly due to portal vein changes that are attenuated by hepatic artery buffering. Reductions in oxygen delivery are shown in both the portal vein and hepatic artery with a concomitant reduction in oxygen consumption despite the increase in oxygen extraction by the liver.

Hepatic response to reoxygenation

Upon reoxygenation, there was an immediate recovery of the THFI compared to baseline and controls, with the PVFI showing greater volume changes than the HAFI. The equivalence of 21% and 100% oxygen in recovery of hepatic arterial perfusion has

been noted in newborn piglet asphyxia using radioactive microspheres,[30] but the greater influence of the PVFI was not evident. We believe that there is no advantage of 100% or 21% oxygen in the immediate restoration of hepatic blood flow after severe hypoxia.

Portal vein DO_2 was significantly greater in the 100% group than either controls or the 21% group, and the total hepatic DO_2 approached significance. The HA DO_2 which did not differ from controls or baseline, thus may have attenuated the excess oxygen delivery from the PV. The PV DO_2 and total hepatic DO_2 in the 100% group recovered to baseline while the 21% group was below baseline at 5 min of reoxygenation phase. This persisted in the 100% group until the change to 21% oxygen, after which the total hepatic DO_2 was below baseline.

There was a gradual increase of hepatic $\dot{V}O_2$ after reoxygenation but 100% oxygen gave no better improvement in oxygen consumption despite the better recovery of DO_2. During the first 60 min of reoxygenation the hepatic EO_2 was lower in the 100% group than the 21% group. It is interesting to note that the decreased EO_2 in the 100% group coincides with the early burst of free radical activity reported in reoxygenation of myocardium[31] and liver.[32]

In newborn piglets, post-hypoxic hyperoxia can lead to a slower recovery of ATP dependent pathways,[33] and of brain hypoxanthine levels.[27] In normal newborn humans, as opposed to post-hypoxic, hyperoxia can allow increased oxygen consumption.[34] We did not observe an increased $\dot{V}O_2$ during hyperoxia in our model of H-R. It is possible that an intermediate oxygen concentration would allow higher consumption than 21% but avoid possible detrimental hyperoxic effects.

Thus, in our model 21% inspired oxygen seemed to be sufficient, with no advantage for 100% oxygen, in restoring hepatic hemodynamics and oxygen consumption. Changes in the portal vein parameters were more marked than in the hepatic artery.

Systemic responses to hypoxia and reoxygenation

Cardiac index in the newborn has been shown to increase over 30-60 min during moderate hypoxemia (S_aO_2 > 40%).[6,7,35,36] Our model, with the S_aO_2 at 41-47%, did not show this response at 60 min of hypoxia, it mimicked a severe hypoxic insult as evidenced by the pH changes.[26,27] The heart rate stability in hypoxia is similar to previous observations.[6,7] The change in the CI, MAP, $\dot{V}O_2$, pH and heart rate from baseline levels in the controls, was not significant during the experimental period, allowing comparison of the observed responses to hypoxia and reoxygenation.

The systemic responses to reoxygenation also confirm previous similar studies.[27,37] Hemodynamic parameters and pH recovered equally with 21% and 100% oxygen. In both H-R groups the CI, MAP and heart rate all recovered within 5 min of reoxygenation and were not different from controls thereafter. The pH remained below baseline and controls in both H-R groups for a similar period of time. The tachycardia compared to baseline, observed in the 21% group after an hour of reoxygenation is not accompanied by changes in other hemodynamic parameters. It did not differ from

The Cori Cycle

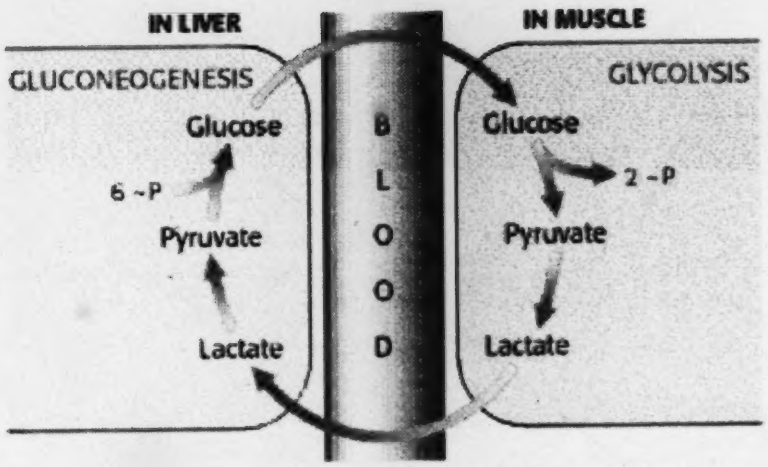

Figure 3-16. The Cori cycle. Lactae formed in muscle or other organs is converted into glucose by the liver. This shifts part of the metabolic burden to the liver.
(From Stryer. L Biochemistry)[43]

the other groups however, so the significance is uncertain. Systemic DO_2 improved up to control levels equally in both H-R groups throughout reoxygenation.

Interestingly systemic $\dot{V}O_2$ remained below baseline in the 100% group until the switch to 21% oxygen, and was also below that of the 21% group at 5 min of reoxygenation. Oxygen consumption has also been reported as impaired in the forebrain of piglets when resuscitation was with 100% rather than 21% oxygen.[38] Thus, our results confirm that there is no advantage of 100% over 21% oxygen in either systemic hemodynamic recovery, acid-base balance or oxygen metabolism in severely asphyxiated newborn piglets.

Lactate metabolism

Lactate has been considered a reliable even though indirect measure of tissue oxygenation,[39] and it has been reported to correlate well with plasma hypoxanthine and base deficit in piglet models of asphyxia.[40] The role of the liver is to convert lactate from the circulation back to pyruvate as part of the Cori cycle (Fig 3-16), hence liver dysfunction may impair resolution of hyperlactatemia. Early in hypoxia the liver has been shown to take up lactate, becoming a net producer later on.[41] In a lamb model, young animals do this more effectively than older ones.[29] However, increased liver tissue lactate in the 21% group suggests that the reoxygenated hypoxic liver cannot metabolize the increased lactate load from other sources in the body despite the apparently adequate DO_2 and improved $\dot{V}O_2$. The clinical significance of this observation is unknown but quantifying the levels of lactate in other tissues may clarify this. The effect of using

Ringer's lactate in the fluid management in this experiment on tissue or circulating lactate levels is also unknown.

Lactate in addition to being a product of glycolysis may be a marker of tissue injury and a predictor of outcome. The arterial lactate level and its resolution is a good prognostic indicator in premature and sick newborn infants, with a higher level indicating a greater likelihood of mortality. A level > 5mmol/l can be associated with over 50% mortality.[42] In liver transplantation an arterial lactate of > 4.0mmol indicates a poor outcome in terms of graft survival,[44] but as this is associated with an ischemia reperfusion insult to a single organ it is likely to be less relevant to global neonatal asphyxia. Lactate levels of >15mmol/l would predict a poor outcome in the more global insults leading to extracorporeal oxygenation therapy in neonates.[45] In our model, the similar plasma lactate in 21% and 100% groups may thus imply a similar long term prognosis in both groups although the level of risk for the lactate levels observed is unknown.

Conclusions

This study supports the conservative use of oxygen, in the resuscitation of asphyxiated newborn infants, for the recovery of hepatic hemodynamics and oxygen metabolism, but notes an associated higher hepatic tissue lactate level when 21% oxygen is used rather than 100%.

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Chapter 4

Liver tissue changes after hypoxia and reoxygenation with 100% or 21% oxygen.

Tissue changes in H-R

Background

Hypoxia and reoxygenation (H-R) injury in the newborn can cause hepatic damage,[1,2] which can be caused by reactive species (free radicals and related compounds) produced in H-R.[3] The newborn may be less able to counter such oxidative stress as they can have low levels of protective antioxidants, such as vitamin E[4] glutathione peroxidase, superoxide dismutase and catalase.[5]

Reactive species arise from several sources. In reoxygenation xanthine oxidase breaks down hypoxanthine, accumulated during hypoxia, to give the reactive oxygen species (ROS), superoxide anion,[6] which can then produce hydroxyl radicals.[7] Endothelial cells and hepatocytes are a source of xanthine oxidase in the liver.[8,9] Nitric oxide (NO) reacts with superoxide to produce the reactive nitrogen species (RNS), peroxynitrite, which nitrosylates tyrosine residues to nitrotyrosine. Furthermore increased intracellular calcium in hypoxia activates phospholipases to produce fatty acid radicals,[3] and mitochondrial electron transport chain dysfunction produces excess ROS.[3]

It is difficult to measure ROS directly in vivo, but peroxidation products and antioxidant levels can be measured and give an indication of oxidative stress. Lipid and protein peroxidation levels increase in proportion to the degree of hypoxia.[10]. Malondialdehyde (MDA) is a byproduct of polyunsaturated fatty acid peroxidation, which has a long enough half-life to be measured. Nitrotyrosine levels give an indication of NO and RNS production. Glutathione is a major intracellular antioxidant and reduced glutathione (GSH) is changed to oxidized glutathione (GSSG) in the presence of ROS or peroxynitrite. The levels of GSH and GSSG can be measured in tissue and the GSSG:total glutathione ratio can be used as an indication of oxidative stress.

The overall energy state of a cell after H-R, may determine whether it survives injury or undergoes necrosis or apoptosis.[11] Hypoxanthine levels are raised in low energy states,[12] in proportion to the duration of hypoxia.[10] Using adenylate levels, the amount of energy available to the cell can be estimated by calculating the energy charge of the cell.[13,14] The serum lactate level is a measure of ongoing oxygen debt. Lactate is produced from the pyruvate arising from glycolysis, in order to regenerate NAD^+, in the absence of oxidative phosphorylation during hypoxia. Higher levels of lactate correlate with longer periods of oxygen deficiency and decreased ATP production.[15]

In the study of liver injury plasma alanine and aspartate transaminase levels give a direct assessment of hepatocyte injury in H-R.[2] Histological examination reveals the degree of vascular congestion, cell necrosis and the degree of infiltration by inflammatory cells.

Liver tissue injury has not been well described in neonatal asphyxia, and the effects of different oxygen levels used in reoxygenation on hepatic injury and oxidative stress are not known. We hypothesized that using 100% oxygen rather than 21% oxygen to resuscitate hypoxic newborn piglets would cause increased oxidative stress and tissue injury without improving the energy status of the cells. Using a newborn piglet model, we assessed hepatic H-R injury using markers of ROS and peroxynitrite production, glutathione levels, levels of liver tissue adenylate nucleotides, hepatic lactate content and flux, plasma transaminases and liver histology.

Methods

The study conformed to the regulations of the Canadian Council of Animal Care (Revised 1993) and was approved by the Health Sciences Animal Welfare Committee, University of Alberta.

Animal preparation and experimental protocol

The animals were prepared as detailed in chapter 3 (p. 91). Following stabilization the animals were randomized to receive hypoxia and reoxygenation with 21% or 100% oxygen. Controls received no hypoxia or reoxygenation as per the protocol in chapter 3 (p. 94).

Blood and tissue analysis

Blood gas measurements (ABL 500 blood gas analyzer, Radiometer, Copenhagen) were performed immediately after sampling. Pre and post hepatic plasma samples for lactate measurement were immediately processed and stored at -80°C until assayed. Immediately after euthanization, a wedge biopsy was taken from the left lobe of the liver and snap frozen in liquid nitrogen for later measurement of tissue metabolites. A section was stored in formalin for histological assessment.

Hepatic energy state and lactate levels

For liver tissue ATP, ADP and AMP measurements, the frozen tissue samples were extracted 1:5 w/v with 6% perchloric acid containing 1mM EDTA. Precipitated protein was centrifuged out (20 min at 20,000xg). The supernatant was neutralized with

3M KOH, 0.4M TRIS, 0.3M KCl. After further centrifugation (20 min 14,000g) the supernatant was assessed with enzyme linked metabolite assays. [17]

The ATP assay was based on the following reactions with absorbance of NADPH measured at 340 nm:

$$\text{ATP + Glucose} \xrightarrow{\text{Hexokinase}} \text{ADP + Glucose-6-P}$$

$$\text{Glucose-6-P + NADP}^{+} \xrightarrow[\text{dehydrogenase}]{\text{G-6-P}} \text{6-P-Gluconolactone + NADPH}$$

AMP and ADP were assessed by the reduction of absorbance of NADH at 340 nm with the following reactions:

$$\text{AMP + ATP} \xrightarrow[\text{Kinase}]{\text{Adenylate}} \text{2ADP}$$

$$\text{ADP + P-pyruvate} \xrightarrow[\text{Kinase}]{\text{Pyruvate}} \text{ATP + Pyruvate}$$

$$\text{Pyruvate + NADH + H}^{+} \xrightarrow[\text{dehydrogenase}]{\text{Lactate}} \text{Lactate + NAD}^{+}$$

The energy charge of the cell was calculated as described by Atkinson. [13,18]

$$\text{Adenylate Energy Charge} = ([\text{ATP}] + \tfrac{1}{2}[\text{ADP}])/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$$

Tissue for lactate assay was prepared as the adenylate samples were. Plasma lactate did not require the tissue preparation prior to assay and samples were also taken at 4 time points during H-R. The assay measured NADH production by absorbance at 340 nm, from the lactate dehydrogenase driven reaction in the reverse direction from the adenosine assays

$$\text{Lactate} + NAD^{+} \longrightarrow \text{Pyruvate} + NADH$$

Hydrazine was added to remove pyruvate and drive the reaction in the desired direction.

The net lactate balance of the liver was calculated

Lactate balance (μmol/min/kg) = Lactate delivery – Lactate output

= [(Arterial lactate mmol/l x HAFI l/min/kg) + (PV lactate mmol/l x PVFI l/min/kg)] – (HV lactate mmol/l x THFI l/min/kg)

Markers of oxidative stress

Tissue response to oxidative stress was measured by ascertaining GSSG and total glutathione levels using a commercially available kit (Glutathione assay kit 703002, Cayman Chemical, Ann Arbor, MI). Liver tissue (50 mg) was homogenized in the kit MES buffer (0.2M 2-(N-morpholino) ethanesulphonic acid, 0.1M phosphate and 2mM EDTA, pH 6.0). After centrifugation at 10,000g for 15 minutes at 4°C, the supernatant was removed and deproteinated. The GSSG is reduced to GSH by glutathione reductase

in the assay cocktail of the kit. GSH combines with 5,5'-dithio-bis-2-nitrobenzoic acid. The absorbance of the yellow product (5-thio-2-nitrobenzoic acid) is read at 405 nm to give a total glutathione level (GSSG + GSH) when compared to kit standards. GSSG was quantified by first derivatizing the GSH in the tissue with 2-vinylpyridine and then assaying as for total glutathione. Total glutathione and GSSG levels were expressed as μmol/g wet weight of liver tissue. The GSSG:Total glutathione ratio of the samples was calculated.

For the MDA assay the frozen tissue was homogenized 1:10 w/v in phosphate buffered 0.9% saline. After combining with 0.8% thiobarbituric acid, fluorescence at 532 nm was compared to standard MDA using Ohkawa's method.[16]

Nitrotyrosine was assayed with a commercially available "sandwich ELISA" kit (Oxis Research™ Nitrotyrosine-EIA, Oxis International Inc., Portland, OR). Liver tissue (500 mg) was homogenized in phosphate buffered saline and centrifuged at 10,000g for 10 min at 4°C. For the assay, a solid phase monoclonal antibody was added to the removed supernatant. An anti-nitrotyrosine, biotin labelled antibody was then added. Streptavidin peroxidase conjugate then binds to the biotinylated antibody and acts on the tetramethylbenzidine substrate to give a yellow colour, read at 450 nm. Quantification was made by comparing to standard dilutions, and results expressed per gram of protein (g prot).

Measurement of serum transaminases

Aspartate and alanine transaminase activities were assayed by enzyme-coupled NADH oxidation, with NADH quantification as above.[17]

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \xrightarrow[\text{transaminase}]{\text{Aspartate}} \text{Oxaloacetate} + \text{Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^{+} \xrightarrow[\text{Dehydrogenase}]{\text{Malate}} \text{Malate} + \text{NAD}^{+}$$

$$\text{Alanine} + \alpha\text{-ketoglutatrate} \xrightarrow[\text{transaminase}]{\text{Alanine}} \text{Glutamate} + \text{Pyruvate}$$

Pyruvate is then assayed in the lactate dehydrogenase reaction described above.

Protein levels

They were measured in the liver tissue against a bovine serum albumin standard for all metabolite results expressed per gram of protein.[19]

Histology

For histological examination the liver tissue was fixed in paraffin, and stained with hematoxylin and eosin. The samples were examined by a pathologist (HI), who was unaware of the experimental group of the samples. Hepatocyte necrosis, vascular congestion and neutrophil infiltration were assessed, using a semi-quantitative scale, modified from that described by Yadav and described in chapter 2 (p. 70) (Table 4- 1 and fig 4-1).[20]

Statistics

Statistical analysis used 2-way ANOVA to compare both inter-group and phase of H-R differences. One way ANOVA with Fisher LSD post hoc was used to examine differences between groups. Non-parametric equivalents were used where appropriate. The threshold for significance was $\alpha < 0.05$. A statistics package (Sigma Stat 2.0, Jandel Scientific, San Rafael, CA) was used. Results are expressed as mean ± standard error of the mean.

Results

Following 120 min of hypoxia, compared to controls, the H-R piglets were hypotensive (mean arterial pressure 56 ± 5, 35 ± 4 and 27 ± 3 mmHg for the control, 21% and 100% groups respectively). They had a decreased cardiac output (108 ± 14, 69 ± 7 and 69 ± 6 ml/min/kg respectively); a decreased hepatic oxygen consumption (0.64 ± 0.14, 0.35 ± 0.09 and 0.23 ± 0.09 ml/min/kg respectively) and were acidemic (arterial pH 7.40 ± 0.02, 7.00 ± 0.04 and 7.00 ± 0.05 respectively) ($p<0.05$, ANOVA). Upon reoxygenation there was immediate recovery of the hemodynamic parameters to control levels and of oxygen delivery to control levels in the 21% group and to 150% of control levels in the 100% group. The pH recovered over 120 min compared to controls, although the 21% group remained mildly acidemic compared to baseline (Table 4-2). The H-R groups did not differ from each other in these parameters.

Hepatic energy state and lactate levels

The liver tissue adenylate charge did not differ between groups (Table 4-3). The arterial plasma lactate rose during hypoxia relative to baseline and controls ($p<0.05$) (Table 4-2). At 120 min of reoxygenation the levels remained significantly higher than controls and baseline, in both H-R groups, but they did not differ from each other at any point. The hepatic and portal venous lactate levels were not different from the respective arterial values at 120 min of reoxygenation. There was no significant net uptake or release of lactate by the liver, in any group, when flux was calculated at 120 min of reoxygenation. The net lactate balance across the liver was 3.9 ± 1.6; 4.5 ± 9.2 and 19.0 ± 31.0 μmol/min/kg in the controls, 21% and 100% groups, respectively. These differences were not significant. ($p = 0.73$, $\beta = 0.95$)

The liver tissue lactate measured from samples after 120 min of reoxygenation, was significantly higher in the 21% group than controls ($p<0.05$), but did not differ from the 100% group (Table 4-3).

Markers of oxidative stress (Table 4-3)

The liver tissue GSSG:total glutathione ratio was significantly greater than controls in the 100% group ($p<0.05$), but did not differ from the 21% group. No differences were noted in the absolute levels of GSSG or total glutathione. Liver tissue MDA and nitrotyrosine levels did not differ between groups.

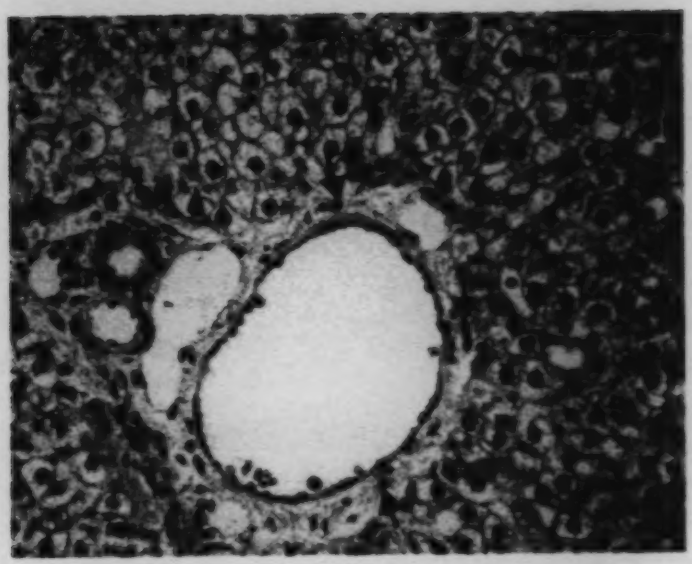
Transaminase activity (Table 4-3)

The plasma levels of liver transaminases did not differ between groups either at baseline or after 120 min of reoxygenation.

Histology

There was no necrosis noted in any sample. Variable degrees of congestion and neutrophil infiltration were noted. Representative sections are illustrated in fig 4-2. Mean congestion scores for controls, 21% and 100% groups were 1.1 ± 0.8, 1.4 ± 0.6, and 1.2 ± 0.5 respectively. Mean neutrophil infiltration scores were 2.1 ± 0.7, 2.6 ± 0.6 and 3.7 ± 0.9, respectively (fig 4-3 to 4-5). There were no significant differences between groups in the individual or the combined histological scores.

A)



B)

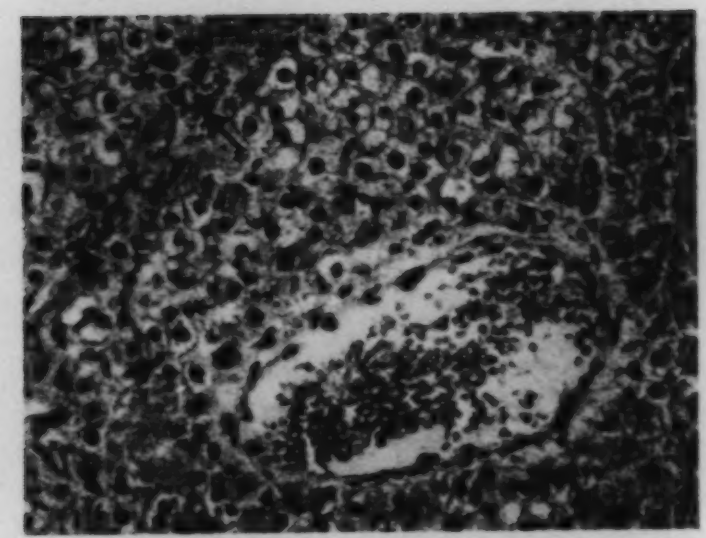
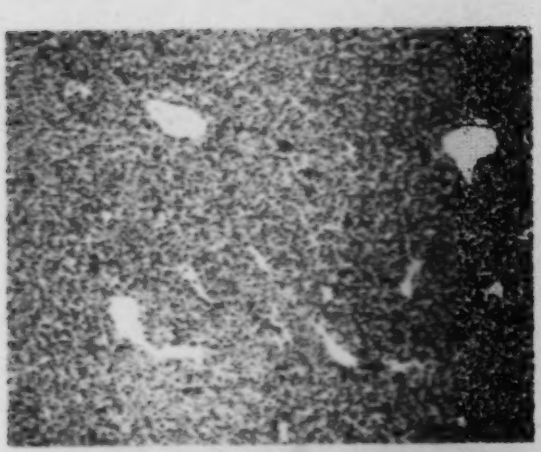
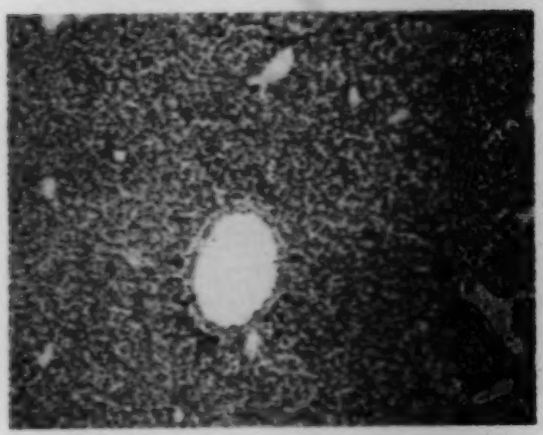


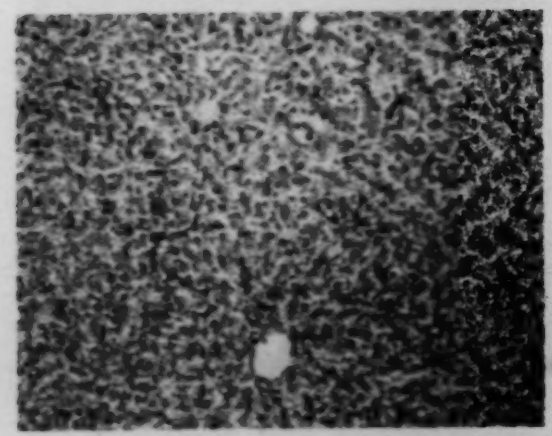
Fig 4-1: H & E stains of liver after hypoxia and reoxygenation illustrative of the features of A) neutrophil infiltration and B) sinusoid congestion. Features are arrowed



Control group



21% group



100% group

Fig 4-2: Illustrative H & E stained post H-R liver for Control, 21% and 100% groups

Score	Extent of zonal tissue necrosis or congestion	Degree of perivenular and periportal zone infiltration by neutrophils	Neutrophil infiltration into intermediate zone (Neutrophils/hpf)
0	<1%	<10%	<1
1	1% - 10%	Mild infiltrate in 11 – 50%	1 – 10
2	11% - 25%	Mild infiltrate in >50%	11 – 20
3	26% - 50%	Dense infiltrate in >50%	21 - 50
4	>50%	Confluent infiltrate in>50%	>50

Table 4-1: Scoring of necrosis, vascular congestion and neutrophil infiltration of post H-R liver. [20]

	0 min Baseline	120 min Hypoxia	15 min Reoxygenation	120 min Reoxygenation
pH				
Control	7.39 ± 0.03	7.37 ± 0.02	7.39 ± 0.02	7.38 ± 0.03
21%	7.40 ± 0.01	7.02 ± 0.04 *†	7.05 ± 0.03 *†	7.26 ± 0.03 †
100%	7.41 ± 0.02	7.00 ± 0.05 *†	7.03 ± 0.04 *†	7.30 ± 0.05
Arterial lactate	(mmol/l)			
Control	1.8 ± 0.2	1.6 ± 0.17	1.5 ± 0.2	2.4 ± 0.22†
21%	1.9 ± 0.16	8.6 ± 0.58 *†	8.0 ± 0.57 *†	7.0 ± 0.82 *†
100%	2.1 ± 0.3	8.5 ± 0.9 *†	7.9 ± 0.9 *†	5.4 ± 1.5 *†
Portal venous lactate (mmol/l)				
Control	-	-	-	2.2 ± 0.3
21%	-	-	-	7.0 ± 1.0*
100%	-	-	-	4.4 ± 1.4
Hepatic venous lactate (mmol/l)				
Control	-	-	-	2.3 ± 0.3
21%	-	-	-	7.0 ± 0.9*
100%	-	-	-	5.6 ± 2.0

Table 4-2: Plasma pH and lactate levels during hypoxia and reoxygenation with 21% or 100% oxygen. Controls did not undergo hypoxia or reoxygenation. ($p<0.05$ * versus controls, † versus baseline) Values are mean ± SEM.

	Controls	21%	100%
Liver tissue lactate	(mmol/g protein)		
	87 ± 17	142 ±16	94 ± 11
Liver adenylate levels	(µmol/g protein)		
ATP	3.1 ± 0.5	2.6 ± 0.3	2.5 ± 0.5
ADP	4.7 ± 1.1	3.2 ± 1.0	2.6 ± 0.8
AMP	6.7 ± 0.7	4.8 ± 0.8	4.5 ± 0.7
Energy charge	0.36 ± 0.05	0.42 ± 0.03	0.37 ± 0.04
Liver glutathione	(µmol/g)		
GSSG	0.05 ± 0.01	0.05 ± 0.02	0.08 ± 0.01
Total glutathione	2.7 ± 0.2	1.9 ± 0.4	2.4 ± 0.2
GSSG:total Glutathione $x10^{-3}$	20 ± 2	28 ± 5	32 ± 3*
Liver peroxidation markers (nmol/g protein)			
MDA	1617 ± 448	733 ± 266	1311 ± 263
Nitrotyrosine	17 ± 2	16 ± 2	20 ± 3
Plasma transaminases	(IU/l)		
AST at baseline	90 ± 17	93 ± 14	84 ± 12
AST at 120 min Reox	62 ± 10	98 ± 25	74 ± 12
ALT at baseline	35 ± 2	36 ± 1	31 ± 3
ALT at 120 min Reox	25 ± 3	22 ± 2	26 ± 4

Table 4-3: Liver and plasma markers of hepatic injury in hypoxia and reoxygenation with 21% or 100% oxygen.
Controls did not undergo hypoxia or reoxygenation. (*p<0.05 versus controls). Values are mean ±SEM. ATP/ADP/AMP = Adenosine tri/mono/di phoshate, Energy charge as defined in methods. GSSG = Oxidized glutathione; MDA = Malondialdehyde; AST/ALT = Aspartate/Alanine transaminase; Reox = reoxygenation

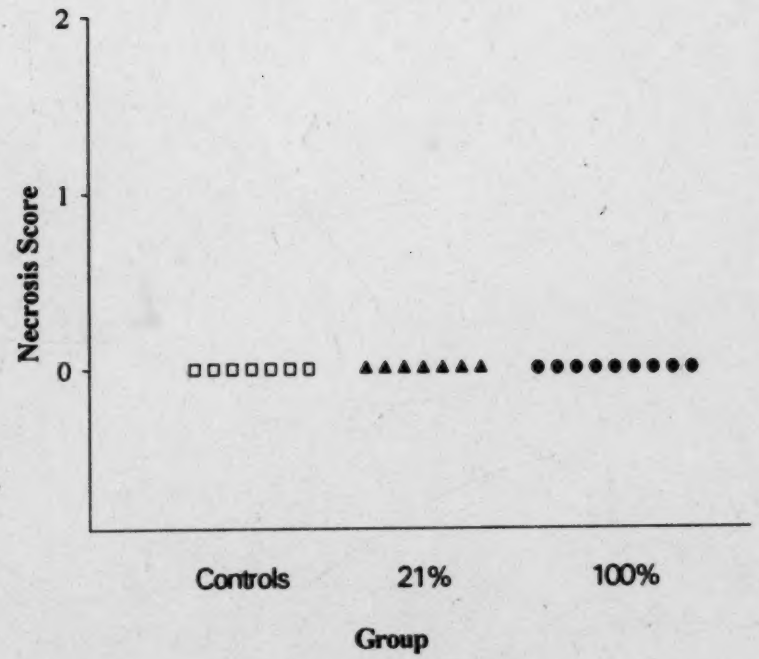


Figure 4-3: Combined zonal histologic scores for degree of necrosis after hypoxia and reoxygenation with 21% or 100% oxygen.

Controls did not undergo hypoxia and reoxygenation. For scoring system see Table 4-1. Two samples were unsuitable for analysis in the 21% group and the control group.

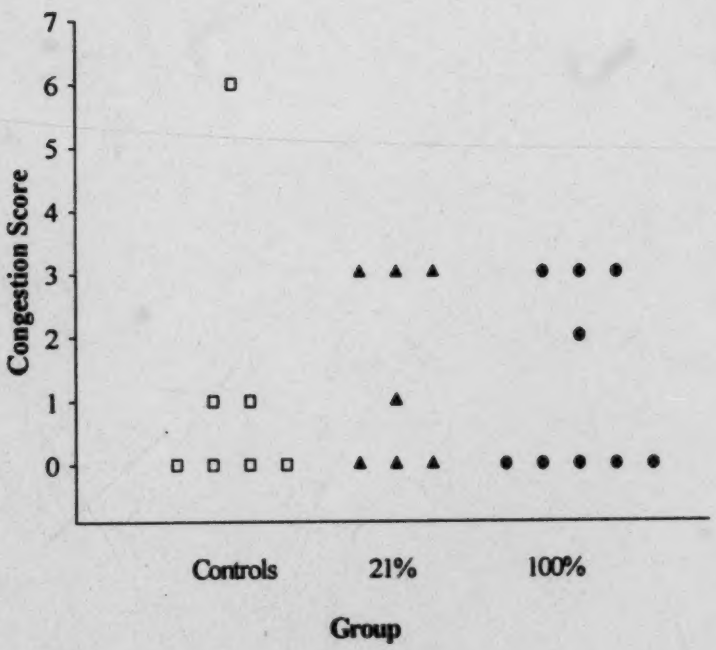


Figure 4-4: Combined zonal histologic scores for degree of vascular congestion after hypoxia and reoxygenation with 21% or 100% oxygen.
Controls did not undergo hypoxia and reoxygenation. For scoring system see Table 4-1. Two samples were unsuitable for analysis in the 21% group and the control group.

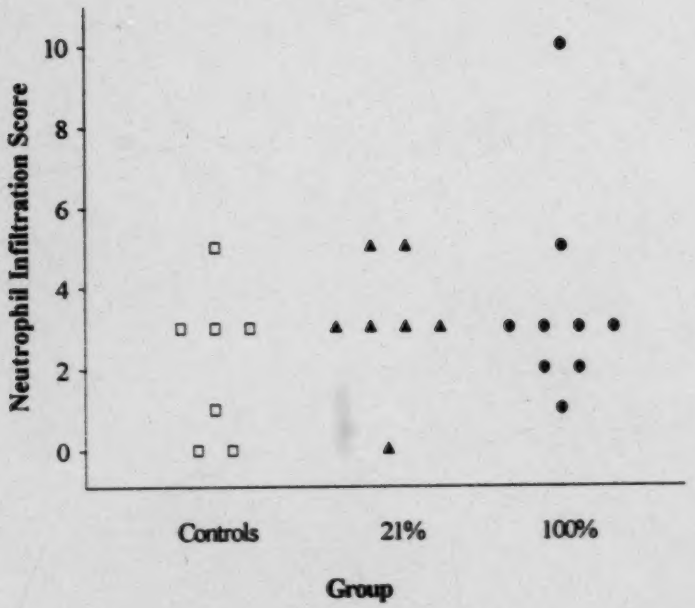


Figure 4-5: Combined zonal histologic scores for degree of neutrophil infiltration after hypoxia and reoxygenation with 21% or 100% oxygen.
Controls did not undergo hypoxia and reoxygenation. For scoring system see Table 4-1. Two samples were unsuitable for analysis in the 21% group and the control group.

Discussion

Our study is the first to examine the liver tissue responses to H-R with 21% or 100% oxygen resuscitation in a newborn animal model.. After 120 min of severe hypoxia and 120 min reoxygenation, liver tissue lactate was elevated in the 21% group but the recovery of liver cell energy charge was independent of the oxygen level at reoxygenation. The consumption of the antioxidant glutathione was significantly increased in the 100% group without a corresponding rise in markers of lipid peroxidation or peroxynitrite production. There were no differences in plasma tranaminases noted and histologic features between the groups, over the timescale of the study.

The degree of metabolic acidemia we achieved seems appropriate for neonatal asphyxia, compared to other piglet models. Cheung et al attained a mean pH of 7.2 after 180 min hypoxia in newborn piglets, with an FiO_2 of 15%.[21] Stonestreet et alin a less severe model in piglets did not reduce the pH below 7.2.[22] Medbo et al achieved a similar pH of 7.0 after 45 – 53 min of hypoxia with an FiO_2 of 8% to our own, and reported the recovery of pH during reoxygenation over a timescale to similar our study, with no difference between 21% and 100% oxygen.[23]

Early in hypoxia the liver has been shown to take up lactate, becoming a net producer later on.[24] We showed that the liver was not a net producer or consumer of lactate at 120 min of reoxygenation in any group. We did not examine the liver lactate metabolism at other time points, and were not able to calculate cumulative uptake during H-R. The role of the liver in the Cori cycle is to convert plasma lactate back to pyruvate, and since the resolution of the plasma lactate is similar in both our H-R groups, with

similar transhepatic gradients we conclude that 21% oxygen is sufficient for the liver to perform this function. We showed higher liver tissue lactate in the 21% group, which may indicate impaired intracellular clearance or increased peripheral production and hepatic uptake, but the clinical or metabolic significance of these findings is not known. It is interesting to note that in resuscitation using <21% oxygen, post-hypoxia, it seems to be the pH recovery that is first impaired as oxygen concentrations are lowered,[25] and we speculate that this may be related to lactate levels.

The adenylate energy charge is a relevant measure to describe the amount of metabolic energy available to the cell.[13,18] With hypoxia or anoxia, ATP production is reduced with concomitant falls in organ ATP content.[26] This reduction serves to preserve the inner mitochondrial membrane potential, which would be dissipated with continued ATP production. The lack of difference between the H-R groups seems to indicate that by 120 min of reoxygenation, in this model, recovery from a presumed deficit during hypoxia seems to have occurred. 21% oxygen is as efficient as 100% oxygen in achieving this recovery. As shown in chapter 3 there was no significant difference between the oxygen concentrations in hepatic oxygen consumption achieved. It is also possible that the oxygen extraction capacity of the liver prevented significant ATP depletion even during hypoxia.

The GSSG:total glutathione ratio showed that giving 100% oxygen resuscitation, caused greater oxidative stress up to 120 min later. Oxidized glutathione has also been shown to be higher in the first 40 min of reoxygenation of anoxic rat hepatocytes,[27] and the effect of systemic oxidative stress can persist for several weeks in infants.[28] The importance of glutathione in liver protection during H-R is not certain,[29] but is an

indicator of oxidative stress. Further research is needed to determine to what degree oxidative stress is damaging to hepatic cells but our short-term transaminase results do not demonstrate significant tissue injury.

No difference between the groups in nitrotyrosine and MDA levels was identified. This may imply equal recovery by 120 min of reoxygenation with both 21% and 100% if abnormal values after reoxygenation are assumed. However the role of nitrotyrosine and MDA as markers of oxidative stress in the liver which has abundant glutathione, remains to be determined. Resolution of MDA levels in a reperfusion scenario can occur within 60 min.[30] In cultured human hepatocytes MDA values have been shown not to rise on reoxygenation,[31] but with in vivo human surgical hepatic ischemia and reperfusion, MDA rises within 30 min.[32] Others have reported maximal levels at 10 min of reperfusion, which is mitigated when Kupffer cells are inhibited.[33] Nitrotyrosine levels also seem to be an effect of Kupffer cell activity. Inhibiting RNS release inhibits the cytotoxic effects of Kupffer cells in hemorrhagic shock.[34] NADPH oxidase seems to be a significant enzyme, as nitrotyrosine levels are greatly reduced in a knockout model after hemorrhage, compared to controls.[35]

The lack of histologic changes in our model is expected given previously reported time scales of inflammatory change in reperfusion settings. Tumor necrosis factor is produced after 1.5-4 h of reperfusion and increased pulmonary capillary permeability and neutrophil infiltration is 3-6 h after reperfusion.[36] After 24 h a pattern of ischemic damage is apparent with hepatocyte loss near the central vein with relative sparing near the portal triad.[37] In more severe insults such as total ischemia from clamping, neutrophil infiltration can be seen as soon as 30 min of reperfusion after 180 min clamping.[38] Our

results, however, confirm that the surgery did not initiate a significant inflammatory response in the model.

It is possible that acute changes in serum transaminases have resolved after 120 min of reoxygenation as this has previously been observed for alanine transaminase,[39] although other studies have shown a more prolonged rise in serum transaminase activity, in clinical cases of perinatal asphyxia.[40] However, the low levels of transaminases may suggest minimal tissue injury as supported by the tissue histology findings. In this model, potential hypoxia induced damage may have been attenuated by the liver's capacity to increase oxygen extraction to maintain cell metabolism. The high GSH content of the liver may also have protected it from oxidative injury.

Sequential biopsies in our model would again allow clarification of tissue lactate, nitrotyrosine and MDA change in the liver during early reoxygenation. Kupffer cell inhibition may assist in identifying which hepatic cell type is having a significant effect.

Conclusions

Following hypoxia-reoxygenation biochemical markers of liver injury and energy charge are similar in the resuscitation with 21% and 100% oxygen. The oxidative stress is higher with 100% oxygen resuscitation, but resolution of liver tissue lactate is slower in 21% oxygen resuscitation. Caution should be exercised in choosing oxygen concentration in neonatal resuscitation, as oxidative stress may be deleterious and the effect of a persisting high hepatic lactate is unknown.

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Chapter 5

Conclusions and Implications

Neonatal asphyxia in the newborn infant causes hypoxia and secondary ischemia with subsequent reoxygenation and reperfusion at resuscitation. Injury caused during and after resuscitation is mediated in part by reactive species. Not all organs are equally affected and injury to one organ may entrain injury elsewhere. The liver seems more resistant to injury than some organs but may nevertheless allow injury elsewhere due to its dysfunction. This research has aimed to identify the possible role of different oxygen levels used at resuscitation on the hemodynamics, oxygen metabolism and tissue changes in the newborn liver, and the presence of reactive species.

We have developed a model allowing the continuous monitoring of hepatic hemodynamics and intermittent assessment of hepatic oxygen metabolism. Due to the development of a novel method of cannulating the hepatic vein, the repeated intermittent assessment of pre and post-hepatic plasma markers was also achieved. Liver tissue assays were possible post-euthanasia. There was a gradual deterioration in portal vein flow and oxygen delivery observed. This may be contributed to by the reduction of hemoglobin, which may have been due to blood sampling or surgical loss. The large amount of intravenous fluid given may also have caused a dilutional effect. However, the relative stability of oxygen consumption suggests that the reduction in delivery was not functionally significant for the liver. Minimizing sampling losses to within the parameters described and meticulous fluid balance when using the model will ensure optimal stability.

In this model of hypoxia-ischemia we demonstrated a reduction in hepatic perfusion and oxygen delivery and consumption during hypoxia. Greater changes occurred in the portal venous system than the hepatic arterial system. On reoxygenation

we showed a rapid recovery of perfusion with both 21% and 100% oxygen. Hepatic oxygen delivery was higher when 100% oxygen was used, but due to reduced extraction the oxygen consumption was the same as that achieved with 21% oxygen. Resuscitation with 100% oxygen causes higher oxidative stress, although a higher liver tissue lactate occurred when using 21% oxygen.

Hence, we confirmed that, with the hepatic artery flow relatively preserved, changes in hepatic blood flow are more dependent on the portal venous rather than the hepatic arterial circulation. The higher oxygen delivery with 100% oxygen allowed greater hepatic oxygen delivery from the portal vein, with similar hepatic artery oxygen delivery, than 21% oxygen.

The hemodynamic and oxygen metabolism observations do not demonstrate an optimal oxygen concentration to use, but we speculate that sufficient oxygen is required but an excess should be avoided.

Lactate flux into and out of the liver was neutral and similar with both levels of oxygen 2 h after reoxygenation. The significance of the raised liver lactate in the 21% group is unknown. The measurement of lactate flux at earlier time points during reoxygenation is needed to clarify the changes in hepatic lactate metabolism. There is no evidence that the higher lactate levels are linked to hepatic tissue damage, however.

The energy charge of liver tissue and the tissue adenylate nucleotide levels were equivalent with 21% and 100% reoxygenation after 2 h of reoxygenation. This would be expected given the equivalence of the oxygen consumption and we speculate that the energy charge at earlier time points in reoxygenation would also be equivalent.

Liver tissue glutathione levels demonstrated a greater degree of oxidative stress when 100% oxygen was used. Measures of lipid peroxidation and nitrotyrosine production did not differ 2 h after reoxygenation, whether 100% or 21% oxygen were used, nor were they different from controls. The liver thus seems to have compensated for the excess oxidative stress from the 100% oxygen supply by consuming antioxidants. This may not be deleterious if sufficient antioxidant capacity remains. The liver has abundant glutathione and is thus protected against high levels of oxidative stress, but this may not apply to other organs.

Histological assessment of hepatic necrosis, vascular congestion and neutrophil infiltration after 2h reoxygenation also showed no difference between the 2 methods of resuscitation.

Some limitations in the model were noted. Despite closing the incisions as much as possible and using wet saline soaks there was an ongoing requirement for fluid infusions above basal requirements. This is a known complication of keeping a laparotomy open for prolonged periods. It was probably a contributing factor to the mild metabolic acidosis, and to the trend for deterioration after 2 hours of reoxygenation. The supply of similar fluid and base to both controls and experiments allowed differentiation of surgical and hypoxic stress. The placement of the hepatic artery flow probe was also technically difficult due to the small size of the vessel, and the common occurrence of anomalous left or right hepatic arteries. If there was no common hepatic artery the dominant artery was used. Umbilical venous catheter positioning has also been a concern in some studies with hepatic damage noted. Our histology confirms this was not a

problem in our model probably due to the placement of the UVC at the junction of the portal vein and umbilical vein by direct vision.

Further work is needed to determine the source of the greater oxidative stress seen with 100% oxygen. Levels of lipid peroxidation and nitrotyrosine need determining in earlier stages of reoxygenation. Additionally, the time course of the experiment did not allow sufficient time to fully compare the inflammatory response in the liver. Measurement of known markers of inflammation in hypoxia and reoxygenation such as TNF-α, at the early stage of resuscitation seen in our model would provide a comparison of the two levels of oxygen without the need to prolong the experiment.

In conclusion, our study confirms the efficacy of using 21% rather than 100% oxygen in resuscitation of the asphyxiated neonate, for the recovery of hepatic hemodynamic and oxygen metabolism parameters. There is no evidence of increased acute tissue damage with 21% oxygen despite a slower resolution of hyperlactatemia and higher hepatic lactate levels. However, the trade off of increased oxidative stress seen with 100% oxygen seems to be well tolerated by the liver.

Our study thus supports the growing evidence that 21% oxygen is at least equivalent to and possibly superior to 100% oxygen in the initial treatment of neonatal asphyxia. Intermediate oxygen concentrations may be appropriate in some cases. Sufficient oxygenation is required and we speculate that the concentration of oxygen required to achieve this may vary between individuals and organs.

Appendix

Fig A-1: Catheterization of the umbilical vein at the porta hepatis

Fig A-2: Ultrasonic flow probes on (a) the hepatic artery and (b) the portal vein

Fig A-3: Intravenous catheter inserted into the left hepatic vein